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TEMPERATURE RELATIONSHIPS AND SOME OTHER CHARACTERISTICS OF THE MICROBIAL FLORA DEVELOPING ON REFRIGERATED BEEF[a]

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(Manuscript received February 12, 1959)

The temperature of storage of meat has been shown to affect the growth of microorganisms. Many of the bacteria which come into contact with meat are mesophiles; these organisms grow poorly at temperatures used for storing meat and may die before conditions are favorable for their proliferation. The selectivity of temperature applies not only to conditions under which the meat is stored but also to the temperature of incubation of media used to isolate and enumerate organisms subcultured from the meat product.

During storage, organoleptic changes, moisture loss and changes in pH occur. The types of organisms isolated from meat stored at refrigeration temperatures has been studied by many workers *(1, 2, 10, 11, 12, 13, 16, 18, 20, 21, 25, 26, 29, 41, 42, 43)*. For example, in 1901, Glage *(16)* reported that the moist surfaces of meat stored at low temperature and high humidity became covered with bacterial colonies. These organisms, which he called "aromabakterien," were oval to rod shaped with rounded ends and occurred occasionally in chains. They were motile aerobes which liquefied gelatin slowly, and turned litmus milk alkaline. They grew well at 2° C but poorly at 37° C; the optimum temperature was thought to be at 10 to 12° C. Glage noted that a characteristic aromatic odor, which he considered rather pleasant in the early stages, accompanied the growth of these organisms. As they grew, the surface of the meat became covered with tiny droplike colonies which increased in size and finally coalesced to form a viscous or slimy coating.

Haines *(22)* concluded that Glage's "aromabakterien" were identical with, or closely related to, the organisms that he thought were responsible for the characteristic slime which appeared on the surfaces of meat stored at low temperatures. Haines *(20)* reported that "with the exception of a certain number of organisms of the *Pseudomonas* group and a few *Proteus*, the bacteria growing on lean meat stored in the range 4–0° C (40–32° F) almost all belong to the *Achromobacter* group." At the same time, but independently, Empey and Vickery *(13)* observed that 95% of the initial flora of beef, capable of growth at −1° C (30.2° F), consisted of members of the genus *Achromobacter;* the remainder were species of *Pseudomonas* and *Micrococcus*. During storage the relative numbers of *Achromobacter* and *Pseudomonas* increased while those of *Micrococcus* decreased. Empey and Scott *(12)* considered the four principal genera of low temperature bacteria isolated from fresh meat and comprising the flora of the carcass to be: *Achromobacter*, 90%; *Micrococcus*, 7%; *Flavobacterium*, 3%; and *Pseudomonas*, less than 1%.

[a] Journal Paper No J-3605 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No 1264.

At present, the general consensus is that the formation of slime on meat is due primarily to multiplication of *Pseudomonas* or *Achromobacter*. In the studies conducted by Haines *(22)*, by Empey and Scott *(12)*, and by Lochhead and Landerkin *(32)*, species of *Achromobacter* were considered to be the predominant organisms. Later Ayres *et al. (5)* and Kirsch *et al. (29)* and Wolin *et al. (43)* reported that species of *Pseudomonas* were predominant. The last three groups of workers recognized that the discrepancy between their results and that of the previous workers probably resulted from changes in nomenclature used in the Sixth Edition of *Bergey's Manual of Determinative Bacteriology* [Breed, *et al.*, 1948 *(8)*] from that adopted in the 3rd Edition [(current at the time that the earlier investigations were conducted), *(7)*]. Recently Brown and Weidemann *(11)* reassessed the taxonomy of the 129 psychrophilic meat spoilage bacteria isolated by Empey and Scott *(12)* and concluded that almost all of these were pseudomonads. Formerly 66 of the stock cultures were listed as *Pseudomonas*, 56 as *Achromobacter* while 7 were unclassified. Empey and Scott previously had allocated organisms to the genus *Pseudomonas* largely on the basis of the production of a water soluble green pigment. Brown and Weidemann pointed out that 21 strains originally classified as pseudomonads on the basis of pigment production, failed to produce pigment in recent tests.

Lochhead and Landerkin *(32)* detected characteristic odors caused by bacteria from New York dressed poultry when counts were in the range of 2.5–100 million per cm^2. The predominant types of bacteria found developing on the skin during storage were identified as representative of the genera *Micrococcus, Flavobacterium*, and *Achromobacter*. Ayres *et al. (5)* identified a characteristic rancid, sweetly aromatic ester-like odor which might be described as a "dirty dishrag" odor which developed when cut-up chickens were stored for a sufficient time at high humidities. The off-odor preceded production of slime and so was considered the incipient sign of spoilage. Shortly following recognition of this off-odor, large numbers of minute, translucent, moist colonies appeared on the cut surfaces and skin of the bird. At first these colonies gave a superficial appearance of being droplets of moisture; however, they became larger and white or creamy in color and often coalesced to form a uniform sticky or slimy layer. A bacterial slime developed on the cut-up eviscerated birds stored at refrigerator temperatures when microbial counts exceeded 100 million per cm^2. In this final stage the meat had a pungent ammoniacal odor in addition to the "dirty dishrag" odor. These indications of spoilage were used as criteria for measuring the storage life of the flesh. Ayres *et al. (5)* using *Bergey's Manual*, 6th ed. [Breed *et al.*, *(8)*] as their taxonomic guide, identified isolates from slimy birds to be closely related to the following species of *Pseudomonas: Ps. ochracea, Ps. geniculata, Ps. mephitica, Ps. putrefaciens, Ps. sinuosa, Ps. segnis, Ps. fragi* or *Ps. multistriata, Ps. pellucida, Ps. rathonis, Ps. desmolytica (um)* or *Ps. pictorum. Bergey's Manual*, 7th ed. [Breed *et al.*, *(9)*] does not list *Ps. sinuosa, Ps. multistriata* or *Ps. pellucida* as distinct species; until the precise taxonomic designation of these three isolates has been determined, their ultimate classification remains in doubt.

Kraft and Ayres *(30)* reported that the first off-odor of fresh sliced beef that they could detect occurred when the surface count approximated 2×10^6

organisms per cm^2; they considered that incipient spoilage was indicated by the onset of off-odor at this level but that definite off-odors were more readily recognized when surface counts on meat samples approximated 10^7 organisms per cm^2.

Ogilvy *(36)* obtained good agreement in comparing either off-odor development or slime formation with a definite bacterial load or with an increase in carbon dioxide production. This is as expected since all three of the methods are manifestations of bacterial growth. It should be recognized that the microbial production of slime is dependent upon the relative humidity of the atmosphere immediately surrounding the beef. According to Scott *(38)* bacterial growth on beef muscle surfaces "only became manifest as slime at relative humidities of 99% and above"; at that time the population was 10^8 or greater. Colonies on meat stored at lower relative humidities failed to coalesce but, instead, remained small, discrete, and almost water-clear.

Various workers refer to certain minimal concentrations of bacteria at the time that meat was slimy to the touch. Haines *(22)*, Empey and Vickery *(13)*, and Schmid *(37)* reported a slime point at populations of 32 million, 50 million, and 50 to 100 million per cm^2 respectively while Moran *(35)* indicated the minimum number of organisms required for sliminess of beef to be 3 million. Kraft and Ayres *(30)* observed slime on sliced beef when loads of organisms exceeded 100 million.

The preceding investigations have shown an association between the growth of certain microorganisms and the production of off-odor and slime. However, some confusion has remained regarding the interrelationships among numbers and kinds of these organisms and the onset of spoilage. The following study is an attempt to evaluate the role of the pseudomonads—and other bacteria—in producing the off-odors and slime which limit storage life of refrigerated sliced beef.

METHODS AND MATERIALS

Fresh beef sliced to a thickness of approximately ½ in was procured from a local retail store at the time of arrival of the carcasses from the processing plant. Both Budget and Choice steaks were studied. These were obtained from, or near, the center of the round. Care was taken that, after cutting, the slices did not come into contact with sources of contamination other than those to which the meat was normally exposed during handling and packaging. After arrival of the packaged meat at the laboratory, pieces 3 x 3 x ½ in were immediately cut by means of a sterile knife, transferred by sterile forceps to a Rodeo [b] peach prepackaging board, packaged in MSAD cellophane or FM-1 Pliofilm and heat sealed.

Meat stored at 0, 5, and 10° C was sampled at 0, 1, 2, 3, 5, 7, 10, 15, 20 and 30 days; that stored at 15, 20 or 25° C was tested at 0, 1, 2, 3, 5, 7, 10, 15 and 20 days.

In general, the sampling procedures were similar to those described earlier [Ayres and Adams, *(3)*]. Swab, rinse, and cut-section samples were compared at 0, 1, 2, 4, 6, 8, and 10 days to determine total numbers of organisms. For swab samplings, absorbent moistened cotton swabs were firmly and thoroughly rolled over the surface in all four directions. Sterile circular aluminum guides having an exposed inner area of 2 cm^2 were used to delineate the sample zone. For rinse samples, meat having a surface area equal to 40 cm^2 was suspended on a sterile mesh screen just above the blades of a Waring blender jar to which 400 ml of sterile water had been added. The blender was turned on for 2 min to allow thorough rinsing of all surfaces before aliquots of the mixture were transferred to petri dishes. For the cut-sections, segments having a 2 cm^2 surface were

[b] Sutherland Paper Co., Kalamazoo, Michigan.

excised with the aid of a sterile cork borer and sterile scalpel to a depth sufficient to yield approximately 2 g of flesh. Another procedure was to undercut 14 x 14 mm blocks of meat to a depth of approximately 11–12 mm; these cubes also contained about 2 g of meat. Each cut-section was carefully weighed on sterile paper and, after weighing, was transferred to a sterile Waring blender or Osterizer jar. For each gram of meat, 99 parts of water were added and the sample blended for 2 min. Serial dilutions of this mixture were used for pour plates in nutrient, trypticase-soy, phenol-red dextrose, malt, violet red bile, brilliant green, fluorescein (King *et al., 28*), thallous acetate (Barnes, *6*) and tellurite glycine agars (Zebovitz *et al., 44*), and in two media devised in this laboratory: nutrient gelatin-K_2HPO_4 and nile blue sulfate lipolytic agar. Anaerobes (facultative and obligate) were recovered by the use of starch thioglycollate agar in Krumwiede-Pratt anaerobic pour plates sealed with a mixture of paraffin and mineral oil.

The composition of the nutrient gelatin-K_2HPO_4 agar was as follows: agar, 15 g; beef extract, 3 g; peptone, 5 g; gelatin, 4 g; K_2HPO_4, 6 g; distilled water, 1000 ml. Nutrient gelatin-K_2HPO_4 plates were used for the detection of chromogenic, fluorescent and gelatin liquefying organisms as well as for total counts. Chromogenic organisms were counted before the reagent for detection of oxidative or proteolytic organisms was applied to the medium. After obtaining total, fluorescent and chromogenic counts, the plates were flooded with 8–10 ml of a solution consisting of 15 g of $HgCl_2$ in 100 ml of distilled water and 20 ml of concentrated HCl; the acid $HgCl_2$ reagent formed a white opaque precipitate with the unchanged gelatin. Oxidase production was determined by pipetting 1–2 ml of a 1% solution of p-aminodimethylaniline monohydrochloride (dimethyl-p-phenylene diamine hydrochloride) on nutrient gelatin-K_2HPO_4 plates. Oxidase positive colonies turned pink and darkened to maroon or black in 6–10 min.

A nile blue sulfate medium for the detection of lipolytic organisms was evolved *(4)* from the procedure proposed by Goldman and Rayman *(17)*. This medium consists of 2 parts: Part A was comprised of an emulsion of lard, water and a monoglyceride emulsifier. The emulsion was prepared by heating 50 ml of distilled water and 15 g of lard to approximately 90° C. This heated mixture was placed in a half pint jar and blended in an Osterizer blender for 1½ min at 110 volts. After blending, 10 g of Armour IV-90 monoglyceride emulsifier were added and the mixture blended for an additional ½ min to produce a paste-like emulsion. Part B consisted of 23 g of nutrient agar (Difco) to which 5 g of agar, 950 ml of distilled water and 50 ml of a 0.1% solution of nile blue sulfate were added. After dissolving the agar base, parts A and B were combined and blended in a 2 quart jar for 1 min using an Osterizer blender at 110 volts. The medium was then bottled, autoclaved at 15 lb pressure for 20 min, cooled and refrigerated until used.

Nutrient agar spread plates were used for colony replication; for these, 0.1 to 0.2 ml of inoculum was placed on the surface of a solidified nutrient agar plate. Often it was necessary to further dilute 1 ml of the customary decimal dilutions of sample with 1–3 ml of physiological saline to provide populations ranging from 30–100 per plate. The plates were rotated and a sterile bent glass rod was used to help spread the material uniformly over the surface. After allowing the surface to dry, the plates were inverted and stored at 20° C. Counts were made after the plates had incubated for 3–4 days. Plates which exhibited well-distributed colonies were chosen as master plates for replica plating [Lederberg and Lederberg, *(31)*]. Imprints of colonies were made on velveteen or Skytex [e] cloth and, from this "negative," reprints were made on all of the solidified agars listed previously.

Since colonies stamped on each medium came from the same master plate, the specific reaction of a given colony in one of the media could be compared to that of the corresponding colony in another medium in order that the predominance of certain types of microorganisms on meat stored at various temperatures could be demonstrated.

Organisms. The colonies were classified on the basis of appearance, amount and type of growth, chromogenesis, proteolysis, lipolysis, acid production, and fluorescence. Individual colonies presenting different morphological or cultural properties were picked from pour plates or spread plates, and subcultures from these were placed on agar slants, in Georgia and Poe's *(15)* asparagin broth, or in nutrient broth. After growth in subculture,

[e] Obtained from Collegiate Mfg. Co., Ames, Iowa.

Gram stains and biochemical screening tests were performed. Motility was determined by microscopic examination of hanging drop preparations of young cultures (16–20 hr) from nutrient broth or by visual observation of diffuse growth spreading from the point of inoculation of isolates grown in SIM medium. Indol and H_2S determinations also were made from this last named medium. The organisms which were indistinguishable by macroscopic appearance, Gram reaction or motility were differentiated on the basis of their activity in gelatin, litmus milk, dextrose, maltose, and fructose media. Reactions were recorded after 1, 2, 4, 7, 10, 14, 30, 45 and 60 days of storage at 20° C. In sugar fermentation tubes phenol red was added as an internal indicator. Occasionally a large number of other biochemical tests were made.

Distinction between oxidative and fermentative production of acid was made from tubes containing dextrose and an internal brom cresol purple indicator by the paraffin layer technique of Hugh and Leifson *(24)*.

Flagella stains were made using Gray's method *(19)*.

Sensitivity to various antibiotics was included since the work of Shewan *et al. (40)* had shown that pseudomonads were insensitive to 2.5 I.U. penicillin and that pigment producing pseudomonads were not sensitive to 10 mcg oxytetracycline. Patterns of sensitivity were determined using standard antibiotic tablets and nutrient agar plates incubated for 1 or 2 days. Whenever possible, the different types of microorganisms were identified in accordance with descriptions given in *Bergey's Manual of Determinative Bacteriology* (Breed *et al., 9)*.

RESULTS AND DISCUSSION

Counts using cut section-, swab-, and rinse-samplings of known surface area were obtained from sliced meat held in a refrigerated display case (Figure 1). The 3 methods gave comparable results. Use of the rinse method was abandoned because of the mathematical considerations involved. Although it was possible to relate weight and surface area to a known volume, this was somewhat tedious and inexact owing to the difficulty of obtaining pieces of meat yielding a total surface area equivalent to 40 cm^2. In general, counts were larger when cut sections of tissue were analyzed than when equal areas of surface were tested; however, results using the swab method followed the same trends as were shown by the more cumbersome cut-section method. For this reason, subsequent tests were restricted to use of swab sampling only.

Growth curves were obtained (Figure 2) for organisms surviving on beef stored at 0, 5, 10, 15, 20 and 25° C. Each of the individual points shown represents an average of 8–10 trials for meat held at that temperature. During the first 5 days of storage, the number of microorganisms recovered was found to vary directly with the temperature of storage. However, by the tenth day the number proliferating at 10 or 15° C was equal to that at 20° C and, by the end of 30 days, the total number of organisms at 0, 5 and 10° C were approximately the same. Slopes for microbial proliferation at 20° and 25° C were about the same; for this reason changes at 25° C are not shown.

Even though the meats tested had very low counts initially, they developed off-odor by the second or third day when held at 25° C, by the third day when held at 20° C, by the fifth day when stored at 10° or 15° C, by the tenth day when held at 5° C and by the 20th day when stored at 0° C. Growth curves of organisms growing aerobically or anaerobically were similar. In either case, a short lag in growth rate was noted at the beginning of the storage period. At the end of the sixth day of storage at 10° C., the number of organisms had reached 50 million per cm^2 and by the seventh day the meat had pronounced off-odor. Slime appeared before the eighth day of storage.

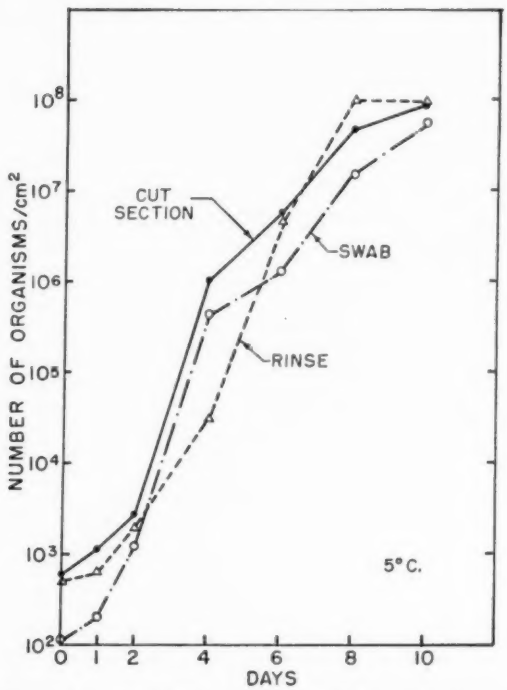

Figure 1. Comparison of growth curves obtained by swab, rinse and cut-section methods of sampling.

At 20° C, spoilage was evident before the fourth day. On samples stored at 5° C, slime usually appeared by the thirteenth day. Meat stored at 0° C developed off-odor and slime at 20 and at 22 days, respectively. Gas production was not noted in the package at this temperature until about 8 days after the formation of slime. When gas was formed, either NH_3 or H_2S could be detected.

After one day of storage the microbial population on meat stored at 5° C was almost invariably less than it was initally and, at 0° C, the number of microorganisms progressively decreased during the first 2 days of storage. Apparently, the nutrients are inadequate for many of the organisms residing on the surfaces of meat or the bacteria are unable to survive at these low temperatures.

That storage at 0° or 5° C is unsatisfactory for a large proportion of the heterogeneous population recoverable initially was further indicated when replicate platings made from meat stored at 15° C were incubated at 5, 15, and 25° C (see Figure 3). The plates stored at 5° C gave an initial count representing only 5–10% of that of plates stored either at 15° C or at 25° C. However, the spoilage types of bacteria—i.e., those associated with off-odor and slime—grew equally well at 5° C, 15° C or 25° C. It is interesting that meat stored for 2 days or longer at 15° C did not yield any significant differ-

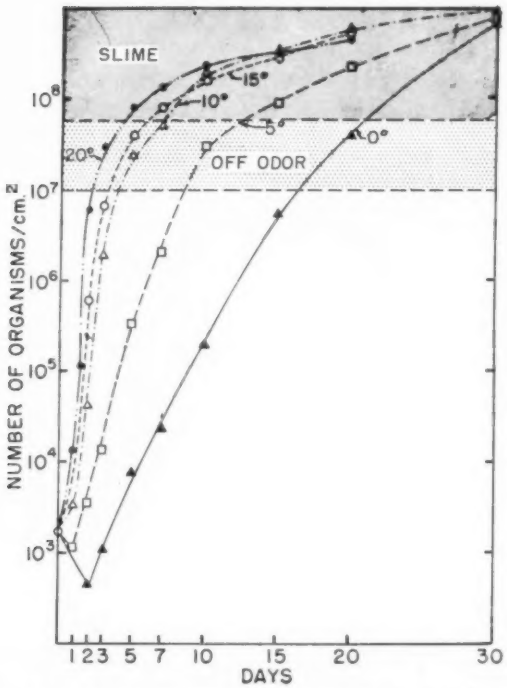

Figure 2. Rates of growth of organisms on beef stored at 0°, 5°, 10°, 15°, and 20° C.

ence in counts whether subcultures were held at 5°, at 15° or at 25° C. Since over 90% of these organisms were species of *Pseudomonas,* they demonstrated equal survival rates at all three temperatures.

Curves in Figure 4 are similar in many respects to those shown by Haines and Smith *(23,* Fig. 3.) However, the 2 sets of figures differ in the following particulars: (1) the data collected by Haines and Smith do not record the reduction in numbers that takes place during the first 1–3 days of storage; (2) the slime point in the present study was set on the basis of visual observations of evidence of sliminess at a value of at least 60 million organisms per cm^2 or a log value of 7.8 (see Figure 2). In the study by Haines and Smith, the point at which slime was definite was considered to be 32 million organisms per cm^2 or 7.5; *(3)* the methods and temperatures of storage were not the same since the meat stored by Haines and Smith was "stored at 0° C in a saturated atmosphere"; that analyzed in this laboratory was placed in packaging film and was held at 5° C.

Figure 5 shows the relation between the initial bacterial load and the time elapsing before slime was observed at 0°, 5°, 10° or 20° C. Also data obtained by Haines and Smith *(23)* for meat stored at 0° C were plotted for comparative purposes. These curves demonstrate that when residential bacterial populations were high, the temperature of storage was less effective in

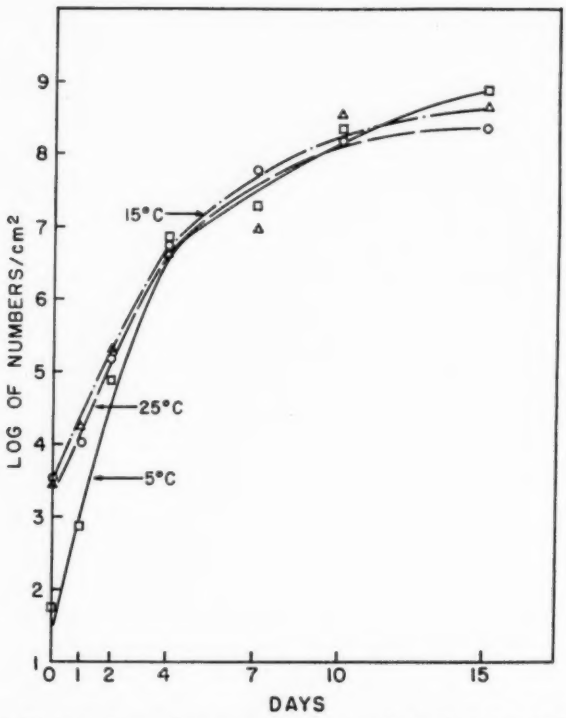

Figure 3. Number of organisms recovered from meat stored at 5° C that develop on nutrient agar plates incubated at 5°, 15° or 25° C.

retarding slime formation than when initial loads were of the order of 50–200 per cm². For example, meat stored at 0° C and containing an initial load of 59,000 organisms per cm² became slimy by the 11th day while that having a starting population of only 65 organisms per cm² did not reach the slime point until the 21st day. At the higher temperatures of storage, initial contamination was of much less importance. For example, meat stored at 20° C with a starting load of 17,000 organisms per cm² was slimy on the third day while that having 140 organisms per cm² reached the same stage of spoilage by the fourth day.

It may be seen in Figure 5 that the two curves for meat held at 0° C do not coincide. It should be noted, however, that the curves have similar slopes and that if Haines and Smith's value for slime had been used (see Figure 4), the time at which incipient sliminess was recorded might have been noted earlier. The amount of moisture that is available to the organisms from the substrate or from its immediate atmosphere has a critical effect (Scott, *38, 39*) as does also the acuity of observation of coalescence of adjoining colonies to form slime. For these reasons it is understandable that results of the present study are at variance with those of the earlier workers.

The replica plating technique was utilized (Table 1) in an attempt to determine the percentage of chromogenic, oxidase producing, gelatin liquefying and lipolytic organisms. Findings using this procedure were not always sufficiently accurate to provide reliable results. Variations in type, size and characteristics of the colonies isolated from meat were such that this method had definite limitations for the isolation and characterization of organisms. Colonies that were hard or dry, small, or more convex or concave than the majority often were not printed. Replication of master plates containing

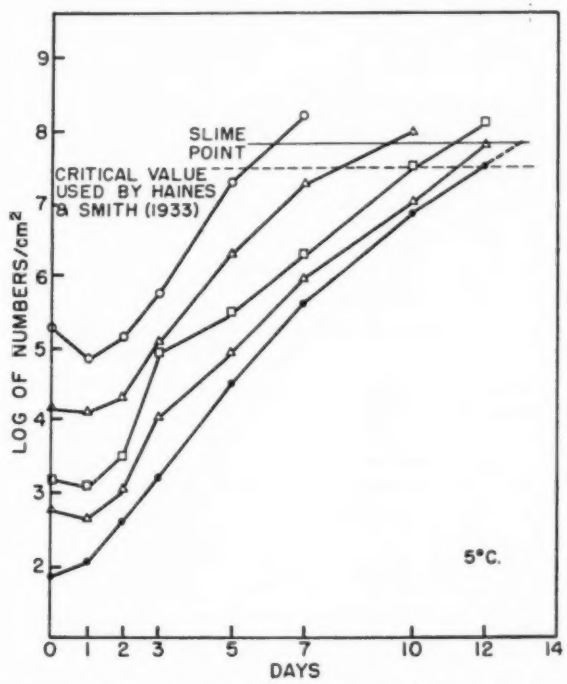

Figure 4. Relation of initial bacterial load to time required for development of slime on meat stored at 5° C.

organisms in excess of 100 resulted in plates that were difficult to read and printed colonies that were not as well defined as the original colonies. Further, when a number of replicates were printed from one master plate, small colonies were not transferred to all the plates from the small amount of material present on the velvet stamp. Also, when large colonies were stamped, uneven elevations allowed the velvet stamp to come in contact with several of the parts but not the entire colony and, as a consequence, several small colonies resulted on the subculture plate. The numbers of colonies observed on the replicated plates in this instance gave erroneous results.

In spite of the handicaps mentioned above, the method was considered of value since the colonies stamped on each medium came from the same master

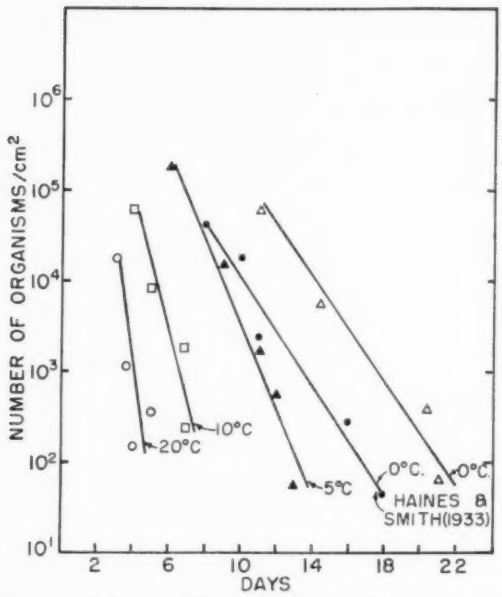

Figure 5. Relation of initial numbers of organisms to the time of appearance of slime on sliced beef held at 0°, 5°, 10° and 20° C.

plate and the specific reaction of a given colony in one medium could be compared to that of the corresponding colony subcultured. With this technique it was possible to demonstrate the predominance of certain types of microorganisms on meat stored at various temperatures. Replica plating also aided in determining the effect of incubation temperature on numbers as well as on types of organisms isolated from meat.

There appeared to be a difference which was related to the temperature of storage in the type of microflora predominating on meat. At 0° C and 5° C Gram negative rods were predominant. Gram positive cocci were the next most numerous. Chromogenic organisms failed to grow as rapidly as did the colorless (white) colonies.

The maximum percentage of chromogens occurred before the meat was stored; sampling indicated 4–28% of the colonies to be chromogenic. After the meat had been refrigerated at 0° C or at 5° C for 4 days, very few chromogenic colonies were recovered. Further, chromogenic colonies decreased in number during the first 4 days at these temperatures. However, it was necessary to store the plates at 15° C or higher in order to recover chromogens.

At 10° C and 15° C there was approximately equal incidence of Gram negative rods and Gram positive cocci. Collectively, 80–85% of the total numbers of colonies were comprised of these organisms. The remainder were other bacilli and chromogenic bacteria.

Results using p-aminodimethylaniline monohydrochloride for the detec-

TABLE 1

Effect of storage time and temperature on total numbers and percentage of chromogenic and fluorescent bacteria found on meats

Temperature of storage of meat		Days of storage									
		0	1	2	3	4	6	8	10	12	14
5° C	Total count (x1000)	4.3		3.4		80	1,220	6,900	31,000	42,000	110,000 [3]
	No. on master plate	43		34		80	61	69	31	42	55
	% replicable	98		97		100	95	97	92	100	96
	% chromogenic [1]	18		8		4	0	1	0	0	0
	% fluorescent [2]	7		9		11	20	13	13	18	19
15° C	Total count (x1000)	4.3	100	560		20,400	83,000	168,000 [3]			
	No. on master plate	43	50	56		102	83	84			
	% replicable	98	98	96		95	94	90			
	% chromogenic	18	11	14		8	0	0			
	% fluorescent	7	16	10		18	15	8			
25° C	Total count (x1000)	4.3	162	10,400	52,000	126,000 [3]					
	No. on master plate	43	81	52	52	63					
	% replicable	98	99	96	92	100					
	% chromogenic	18	20	15	4	1					
	% fluorescent	7	13	9	26	4					

[1] Chromogenic colonies detected on nutrient gelatin-K_2HPO_4.
[2] Fluorescent colonies detected on the medium of King *et al.* (*28*).
[3] Meat slimy.

tion of oxidase production were not reliable. The reagent was unstable and the test was difficult to read. Often large variations in numbers of oxidase producing colonies were noted. Occasionally, duplicate colonies did not give identical results. Also the reagent was found to be unstable to light. Care was necessary in providing the proper exposure time before reading reactions; for some colonies this may have been insufficient and for others excessive. Initially about 25% of the colonies gave an oxidase reaction but the percentage gradually increased with storage time and by the time that the meat had reached the slime point, most of the colonies gave a positive reaction.

The numbers of organisms able to liquefy gelatin fluctuated greatly within and between samples. From 35–50% of the organisms found initially on beef demonstrated the presence of gelatinase. However, the replica plating technique was not considered sufficiently sensitive to detect the exact number of organisms having this ability. In several instances, some of the colonies were surrounded by such large clear zones that negative reactions by neighboring colonies were obscured. Also, differences in the percentages of colonies displaying gelatinase activity during storage varied so greatly that, except for the general observation that from one-third to two-thirds of the colonies possessed gelatinase, no other specific pattern could be determined.

The medium suggested for detecting lipolytic organisms (Fanelli and Ayres, *14*) was pretested with known lipolytic and non-lipolytic organisms. This medium was preferred to that proposed by Goldman and Rayman *(17)* since it was much easier to prepare. The medium could be autoclaved and did not deteriorate during storage at refrigeration temperatures.

Lipolysis was determined by the presence of a blue colony; the medium itself was a pale blue to lavender in color. Colonies ranged in color from white to deep blue. In addition, strongly lipolytic organisms produced a blue zone 5–10 mm in width surrounding the colony. Non-lipolytic bacteria appeared as white or as very pale, blue-centered, white colonies. Some of the colorless colonies were surrounded by pink zones. Plates incubated at 15° C produced positive reactions in 4 days; similar results were obtained at 20 or 25° C within 3 days. Several strongly lipolytic strains of *Pseudomonas fragi* gave equal growth on this medium and on nutrient agar. However, in tests utilizing strains of *Staphylococcus aureus, Micrococcus flavus,* and *Sarcina lutea,* the Nile blue sulfate dye was found to be inhibitory. One strain each of *Staphylococcus aureus* and *Sarcina lutea* produced only about one-tenth the number of colonies that were noted on nutrient agar plates.

Jones and Richards *(27)* suggested the use of Victoria blue, a triphenylmethane dye, as a lipolysis indicator. When Victoria blue was used instead of Nile blue sulfate, the medium was less inhibitory for pure cultures of micrococci but, when a mixed flora of the meat was tested, little difference was noted in the numbers of lipolytic organisms isolated on the two media.

The temperature of storage of meat did not seem to affect the lipolytic activities of the organisms isolated. Usually about 75–90% of the organisms present were able to utilize lipid materials regardless of the storage temperature.

When replica agar plates were prepared from meats stored at 15° C and incubated at different temperatures (5° C, 15° C or 25° C) lipolytic and gelatin liquefying activities were similar.

There was a heterogeneous population of microorganisms on fresh beef (Table 2). For the most part chromogenic organisms were identified as representatives of the genera *Flavobacterium, Micrococcus, Sarcina* and *Serratia.* Less than 1 to approximately 10% of the colonies were yeasts and molds; 2–40% were colorless micrococci, streptococci, "lactobacilli," spore forming aerobes and anaerobes, and miscellaneous bacteria. The remaining non-pigmented organisms were *Pseudomonas* and *Achromobacter* (15–45%).

TABLE 2

Classification of bacteria, actinomycetes, molds, and yeasts isolated from refrigerated beef

Bacteria and Actinomycetes [After Bergey's Manual of Determinative Bacteriology, 7th Ed. *(9)*]	*Molds and Yeasts*
Schizomycetes	Phycomycetes
Pseudomonadales	Mucorales
Pseudomonadaceae	Mucoraceae
Pseudomonas	*Rhizopus*
Aeromonas	*Mucor*
Eubacteriales	Thamnidiaceae
Achromobacteriaceae	*Thamnidium*
Alcaligenes	Fungi Imperfecti
Achromobacter	Moniliales
Flavobacterium	Moniliaceae
Enterobacteriaceae	*Monilia*
Escherichia	*Aspergillus*
Aerobacter	*Penicillium*
Paracolobactrum	*Sporotrichum*
Serratia	Dematiaceae
Proteus	*Cladosporium*
Salmonella	*Alternaria*
Micrococcaceae	Cryptococcales [After Lodder & Kreger-van Rij (33)]
Micrococcus	Cryptococcaceae
Staphylococcus	*Torulopsis*
Sarcina	*Candida*
Lactobacteriaceae	*Rhodotorula*
Streptococcus	
Corynebacteriaceae	
Microbacterium	
Bacillaceae	
Bacillus	
Clostridium	
Actinomycetales	
Streptomycetaceae	
Streptomyces	

It was not always possible to differentiate between *Pseudomonas* and *Achromobacter* since the two genera have similar morphology with the exception that pseudomonads have polar flagella. However, with the use of two or more of the following criteria, most of the isolates were ascribed to the genus *Pseudomonas:* (1) polar flagella, (2) fluorescent pigment, (3) oxidative production of acid and (4) lack of sensitivity to penicillin (while 2.5 I. U. was specified by Shewan *et al. (40)*, 5.0 I.U. was ordinarily used since it was found that other organisms as well as pseudomonads were able to survive at the lower concentration. 5.0 I.U. did not inhibit the pseudomonads).

Brown and Weidemann *(11)* have advocated the use of the methods of Hugh and Leifson *(24)* for characterizing oxidative, as opposed to fermentative, production of acid and those of Shewan *et al. (40)* for determining sensitivity to penicillin; these procedures were also included for the further identification of the *Pseudomonas*.

Organisms belonging to many different genera were identified (Table 2) as occasional contaminants of meat, but only *Pseudomonas, Achromobacter, Flavobacterium, Micrococcus, Microbacterium,* and *Penicillium* were regularly found from examinations made of a large number of isolates from refrigerated sliced beef.

The relative numbers of the several organisms which were recovered are of considerable interest. *Pseudomonas* and *Micrococcus* were predominant at zero time and throughout the storage period. However, at the time the meat was received, organisms including *Achromobacter, Flavobacterium,* spore forming organisms, chromogenic bacteria, molds and yeasts constituted 20–50% of the total population. Molds were uncommon on fresh beef at the time it was received.

There appeared to be a relation between the type of flora developing on meat and the temperature of storage. Meat kept at low temperatures for several days had a restricted bacterial flora consisting primarily of *Pseudomonas* and *Micrococcus*. Pseudomonads predominated on meat kept at 0° C, 5° C or 10° C while at 15° C or higher there was approximately an equal incidence of pseudomonads and micrococci. There were occasional organisms representative of the genera *Achromobacter, Flavobacterium, Bacillus, Clostridium* and *Streptococcus,* but the enterobacteria and staphylocci were not recovered from meat after several days of storage at temperatures below 10° C. One strain of *Streptomyces* was isolated from a lot of beef that had had the packaging material torn while in transit from the processing plant. Two samples of meat showed the presence of streptococci while from one a diphtheroid was recovered.

Species of *Penicillium* were more commonly isolated from beef than were any other molds. *Cladosporium, Thamnidium, Mucor* and *Rhizopus* were next in order. *Aspergillus* was encountered occasionally on meat held at temperatures of 10° C or higher. Also, two separate isolations of *Monilia* and single isolations of *Alternaria* and *Sporotrichum* were made. For the most part, the activity of molds was restricted to the outer surfaces where aerobic conditions were maintained. On samples of meat that had been refrigerated for several days, *Penicillium* and *Cladosporium* often were found growing on the connective tissue or on the native layer of fat covering the muscular tissue. Either of these organisms produced spots ranging in color from yellow to black although discolorations attributed to *Cladosporium* ordinarily were darker in color than those identified as *Penicillium*.

Strains of organisms belonging to three asporogenous genera of yeasts—*Torulopsis, Candida,* and *Rhodotorula*—were occasionally recovered from refrigerated meat; no sporogenous yeasts were isolated.

In freshly sliced meat or in that stored at 15° C or higher, species of *Micrococcus* and *Flavobacterium* were abundant but the predominant flora at the time that the meat developed off-odor or became slimy was comprised of motile Gram negative rods occurring singly, in pairs, or in short chains.

These almost invariably were found to be pseudomonads. In rare instances strains of *Achromobacter* and *Alcaligenes* were recovered from meat at the time of off-odor development. Also, on one occasion a polar flagellated organism was isolated which attacked glucose fermentatively. This agrees with the observation of Brown and Weidemann *(11)* that one of the organisms isolated by them was possibly a species of *Aeromonas*.

Pseudomonads recovered from refrigerated sliced beef were related to known species of the genus. Table 3 shows these relationships. Of the isolates, organisms that might be classified as *Pseudomonas geniculata* were by far the most frequently encountered species and were followed in order of prevalence by strains similar to *Ps. fragi* and *Ps. rugosa*. The observations agree with the findings of Kirsch *et al. (29)* and Wolin *et al. (43)*. In addition, Wolin *et al.* concluded that, except for the gelatin test, "about 90% of the isolates were either *Ps. geniculata* or differed from it primarily by an inability to liquefy gelatin."

TABLE 3

Relation of organisms isolated from refrigerated sliced beef to known species of Pseudomonas

Strain no	Species given in Bergey, 7th Ed. *(9)*	Differences from reported species
P-92	*Pseudomonas fluorescens*	
P-65	*Pseudomonas geniculata*	Sugars not reported.
P-80	*Pseudomonas syncyanea*	
P-43	*Pseudomonas putida*	
P-51	*Pseudomonas striata*	
P-16	*Pseudomonas taetrolens* or *Pseudomonas rugosa*	Some growth at 37° C. Indole not formed; has musty odor.
P-37	*Pseudomonas convexa*	
P-62	*Pseudomonas fragi*	Alkaline in maltose and fructose not reported.
P-58	*Pseudomonas mephitica*	Skunk-like odor in 1–2 days, and slow liquefaction of gelatin reported.
P-7	*Pseudomonas putrefaciens*	Rapid reduction of litmus reported.
P-13	*Pseudomonas rathonis*	Maltose and fructose not reported.
A-76	*Pseudomonas ochracea*	Grows well at 25° C.
A-90	*Pseudomonas segnis*	
P-26	*Pseudomonas* sp.	Similar to *Ps. sinuosa* reported in Bergey, 6th ed. but not given in 7th ed.
P-34	*Pseudomonas* sp.	Gelatin liquefied; acid and gas from glucose; litmus milk coagulase.

Pigment production was an elusive diagnostic criterion. The ability of some of these organisms to produce pigment was associated with the temperature of the storage of the meat and of the plates during subculture. In many instances pigment was not observed until plates which had been stored at 5° C were allowed to remain at room temperature for an hour or two. Also pigment production seemed enhanced by permitting more aeration (by slightly lifting the petri dish cover for an hour or two before reading the plate). In addition, some of the organisms lost the ability to produce pigment when subcultured.

Small, generally Gram positive, non-motile rods were tentatively classified as "lactobacilli." However, since the isolates were catalase positive and

varied in length from short rods to filamentous forms, the possibility was considered that they might be members of any of the following genera: *Corynebacterium, Microbacterium* or *Arthrobacter.* These organisms were quite short (0.5 to 0.7 by 1.0 to 2.0 mc), and Gram variable. The question of their precise identification is both puzzling and intriguing. Kirsch *et al. (29)* isolated Gram positive, non-motile rods from hamburger but, since those organisms were catalase negative, they determined them to be species of *Lactobacillus.* Except for the catalase reaction, organisms isolated in the present studies had similar biochemical patterns to those reported by Kirsch. The following characteristics suggested placement in the genus *Microbacterium* rather than *Corynebacterium* or *Arthrobacter:* the production of catalase, ability to hydrolyze starch, production of acid but no gas from glucose, fructose, mannose, maltose, and litmus milk, failure to reduce nitrates, coupled with the observation that the cultures grew fairly well aerobically. These organisms would be considered identical with *M. lacticum* except that they usually did not ferment lactose and they failed to survive temperatures higher than 60° C. for 30 minutes. McLean and Sulzbacher *(34)* isolated a somewhat similar organism from fresh pork sausage; they suggested the name *Microbacterium thermosphactum* for this organism. Brown and Weidemann *(11)* found 7 of 189 psychrophilic meat spoilage bacteria to be Gram positive. They considered that 4 of these were probably species of *Corynebacterium.* In the present study, only one diphtheroid was recovered. This organism was lost before it could be positively identified and so it is not known if it was classifiable as a *Corynebacterium, Microbacterium* or *Arthrobacter.*

A few species were coccoid in character. Although none of the organisms isolated were thought to belong to the genus *Leuconostoc,* there remains some doubt that *Leuconostoc* can always be separated from some types of *Lactobacillus* by morphological and cultural characteristics.

SUMMARY

Growth curves were obtained for numbers of organisms surviving on beef stored at 0°, 5°, 10°, 15°, 20° and 25° C. These were related to the development of off-odor and slime. It was shown that the initial load of organisms determined the time required for development of slime. The critical value for slime production was found to be 6×10^7 organisms or a log value of 7.8. There was a change in slope for the effect of temperature at 0° C when compared with that of 20° C.

Comparison was made of the swab, rinse and cut-section methods of sampling; swab samples were selected as being the most reproducible and most convenient to use.

The effect of storage time and temperature on percentages of lipolytic, gelatin liquefying, oxidase producing, chromogenic and fluorescent bacteria was studied. Most of the bacteria on stored meats had lipolytic activity; from one-third to two-thirds of the organisms liquefied gelatin. Oxidase production could not be determined accurately. From 4–20% of the initial population were found to be chromogens but colored colonies were rarely found beyond the fourth day of storage; also, plates stored at temperatures less than 15° C did not yield chromogens. Fluorescent bacteria comprised 10–25% of the flora throughout storage.

Microorganisms isolated from refrigerated beef were classified and it was found that at 10° C or lower the bacteria responsible for production of slime were almost without exception pseudomonads. Various species of *Pseudomonas* were identified. At 15° C or above, there was approximately an equal incidence of organisms of the genus *Micrococcus* and *Pseudomonas*. Other bacteria that were recovered from the sliced meat were *Achromobacter, Flavobacterium, Microbacterium, Alcaligenes, Aeromonas, Bacillus, Clostridium* and *Streptococcus*. Species of *Penicillium* were the most commonly encountered molds, more rarely, *Cladosporium, Thamnidium, Mucor, Rhizopus, Aspergillus, Alternaria*, and *Sporotrichum* were found.

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SPOILAGE OF VACUUM-PACKED SLICED PROCESSED MEATS DURING REFRIGERATED STORAGE [a]

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Processed meat products are commonly stuffed and cooked in relatively impermeable casings and are not subject to internal contamination during handling subsequent to cooking. Spoilage of such products can be caused, therefore, only by organisms that survive the process or by contaminants growing on the surface. Jensen *(4)* has reviewed much of the work on the flora to be found in such products. Steinke and Foster studied qualitative and quantitative changes in the bacterial flora of liver sausage and bologna during prolonged refrigerated storage *(10, 12)* and determined the influence of the casing on spoilage *(11)*.

The current practice of slicing sausages and marketing them in consumer-sized packages has introduced the opportunity for additional contamination during preparation for marketing. Commercial practice has shown that these products will undergo bacterial spoilage in time, the spoilage usually taking the form of a slimy surface and, sometimes, green discoloration. The latter defect is noticeable only when oxygen reaches the surface of the meat, either by diffusion through the wrapping or through a leak.

Niven and his co-workers *(5, 6, 7)* found that heterofermentative lactobacilli and leuconostocs were associated with spoilage of processed meats. The organisms were isolated from meat samples showing green discolorations collected from a large number of sources throughout the United States. Steinke and Foster *(10)* also noted that lactic acid bacteria, although not detected in freshly-made liver sausage, were the predominant organisms after several weeks' storage at 41° F (5° C) when the product was stuffed in Visking cellulose casing and overwrapped with Pliofilm.

From these and other studies it was anticipated that lactic acid bacteria might be involved in the spoilage of sliced processed meats packaged under vacuum. Therefore, provision was made in this study to detect lactics if they were present. The study involved holding commercial packages of several representative types of processed meats under refrigeration and observing the changes in appearance and bacterial flora. Organisms were isolated at intervals for identification of the predominant types.

METHODS

Meat samples. Commercial, half-pound, vacuum-packed, sliced meat products of 4 different types, each from a single production lot, were obtained as they came off the production line. Samples from a single lot were assumed to have essentially the same

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degree of contamination and would afford maximum replication among packages for storage tests. The 4 products were "Bologna", "Chopped Pressed Ham", "Smokie Patties", and "P & P Loaf." All were produced in the same packing plant.

These products were used because there are significant differences in their composition and treatment that might influence spoilage. Bologna represented a water cooked product that was mildly spiced. P & P Loaf was of the same general type but contained chopped pickles and pimientos. Chopped Pressed Ham contained more salt and was composed of larger pieces of meat than the other products. Smokie Patties consisted of a smoked sausage prepared in the form of patties. The first 3 products were sliced from large loaves.

Each package consisted of a metal base and a Saran plastic film which covered the meat. The film was attached to the base with a heavy plastic strip.

Samples of each product were stored at 34° F (1.1° C) and at 45° F (7.2° C). These temperatures correspond to ones used in retail stores and in the home respectively.

Sampling and plating. A bacterial count was made on each product the day it was obtained and at 5 to 10 day intervals on samples stored at each temperature until spoilage was apparent.

Three packages chosen at random were used for each plating. Each was opened aseptically and a section about one-half inch wide was removed by slicing across the center of the stack of slices with a sterile knife. Thus a portion of each slice was obtained. The sample was adjusted to 20 g by trimming along the edge of the strip and then minced in a sterile Waring blender bowl with 180 ml of sterile water. Further dilutions were made in sterile physiological saline and plated in triplicate in APT agar *(2)*. Plates were incubated aerobically at 30° C for 3 days. The results for the 3 packages (nine plates) were averaged to obtain the plate count per gram of meat.

At the time of plating each sample was examined for taste, odor and visible slime.

NaCl and water content of the meat. The quantity of NaCl and water in the meat was determined at the beginning and again at the end of the storage period. The method of VanSlyke and Price *(13)* was used for the salt determinations; the moisture content was measured by drying samples to constant weight in a vacuum oven at 100° C *(1)*.

Isolation of bacteria. Twelve colonies were picked at random from the countable dilution of each set of plates into APT broth. The 606 isolates thus obtained were observed for morphology, Gram reaction, spore formation, and catalase production. Those retained for further study were carried as stab cultures in APT agar containing 0.2% glucose.

More detailed characterization of representative isolates

Reduction of nitrate. Tubes of APT broth containing 0.1% KNO_3 were inoculated with young cultures, incubated 1 week at 30° C, and tested for nitrite with α-naphthylamine and sulphanilic acid.

Growth on nutrient agar slants. Young cultures were inoculated onto nutrient agar slants, incubated at 30° C and examined for growth after 24, 48 and 168 hours.

Optimum growth temperature. Tubes of APT broth inoculated with a young culture of each isolate were incubated at 45, 37, 30 and 15° C. The cultures were observed periodically for 2 weeks for turbidity.

Fermentation of carbohydrates. Solutions of the desired sugars and alcohols were sterilized by Seitz filtration and added to Durham tubes of APT broth from which the phosphate and glucose were omitted. Brom cresol purple was used as an indicator. The final concentration of substrate in the medium was 1%. The tubes were inoculated with young broth cultures and examined for acid and gas production at 24, 48 and 168 hours.

Hetero- or homofermentation of glucose. The methods and medium of Gibson and Abdel-Malek *(3)* were used. The cultures were incubated at 30° C and examined for gas production at 48 and 168 hours.

Oxygen requirements. Shake cultures in APT agar were examined for location of growth after 24, 48 and 168 hours.

Growth at pH 9.6. APT agar adjusted to pH 9.6 with sterile NaOH after autoclaving was inoculated by stabbing. The cultures were incubated at 30° C and examined for visible growth at intervals for one week.

NaCl tolerance. APT broth with varying concentrations of NaCl were inoculated, incubated at 30° C and examined for growth at 24, 48 and 168 hours.

Greening of wieners. Wieners were quartered, steamed for 10 min at atmospheric pressure and placed aseptically in sterile Petri dishes. Each of 2 quarters was inoculated with 0.5 ml of a young culture. The wieners were examined after 2 and 7 days' incubation at room temperature for signs of greening.

Thermal death-time studies. The methods of Olson *et al. (9)* were adapted for the present work. A 24-hour culture in APT broth diluted to contain 50,000 to 200,000 cells per ml was used for all heating tests. The final dilution was made in APT broth. Two ml quantities of this inoculated broth were transferred to sterile Pyrex culture tubes (10 x 75 mm). Ten tubes were used for each heating period. Each tube was sealed in an oxygen flame so that complete immersion was possible. The sealed tubes were heated for the appropriate time in a constant temperature (±0.05° C) water bath, then cooled at once in ice water. The tubes were incubated at 30° C and examined periodically for one week for visible growth. The thermal death time at a given temperature was considered to be the minimum time required to render the bacteria in a set of 10 tubes incapable of growing.

RESULTS

Figure 1 illustrates the changes in the meat products during storage. All samples maintained their normal color and odor, although some developed atypical flavors and a visible, gummy slime. The slime was detectable when the packages were opened and the slices were pulled apart. The occurrence of these defects was directly related to the rate and amount of bacterial growth, which in turn varied with the product and

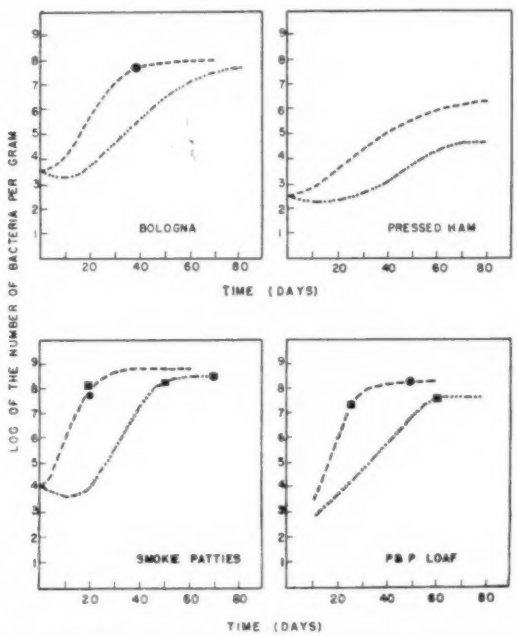

Figure 1. The effect of temperature and time of storage on the number of organisms found in the meat samples.

– – – – – storage temperature: 45° F (7.2° C). – · · · – · · storage temperature: 34° F (1.1° C).
■ Time at which slime was first noticed. ● Time at which the flavor became atypical.

with the temperature of storage. Signs of spoilage were detectable, if they appeared at all, after the bacterial population exceeded 10,000,000 per gram.

Smokie Patties and P & P Loaf supported the most rapid bacterial growth and, consequently, most rapid spoilage. With these products visible slime appeared before any changes detectable by taste. Pressed Ham, on the other hand, supported relatively little bacterial growth and showed no evidence of spoilage. Bologna was intermediate between Pressed Ham and the other products. Bacteria grew much more slowly in Bologna than in Smokie Patties, for example, but the final populations in Bologna were in the neighborhood of 100,000,000 per gram. This product did not become slimy, although atypical flavors were noticed in the samples stored at 45° F (7.2° C) after 40 days.

The advantage of the lower storage temperature in retarding spoilage is obvious with all of the products. Samples stored at 34° F (1.1° C) remained normal for at least 30 or 40 days longer than corresponding samples held at 45° F (7.2° C).

When they were first placed in storage all 4 products contained only a few hundred to a few thousand organisms per gram. Except for a single isolate from Smokie Patties, all of these were aerobic and catalase positive. Cocci were in the majority, although rods were found in all samples and a few yeasts were isolated from all products except Bologna. These results are in general agreement with those of Steinke and Foster *(12)* for liver sausage and bologna.

Catalase negative organisms rapidly became predominant as growth occurred. Table 1 illustrates the shift from a catalase positive to a catalase negative flora in each product at each temperature. As would be expected the change occurred most rapidly in products and at temperatures that permitted most rapid growth. All of the catalase negative isolates were Gram positive nonsporeformers and, from all products except Pressed Ham, over 99% of them were rods. From the ham, however, only 82% of the organisms were rods.

TABLE 1

The change from catalase positive to catalase negative flora during storage

Storage		Percent of isolates							
		Bologna		Pressed ham		Smokie patties		P & P loaf	
Temperature	Time	Cat+[1]	Cat−[2]	Cat+	Cat−	Cat+	Cat−	Cat+	Cat−
	(days)								
	0	100	0	100	0	90	10	...[3]	...[3]
	10	15	85	100	0	35	65	0	100
45° F	20	0	100	45	55	0	100	0	100
	30	0	100	40	60	0	100	0	100
	40–70	0	100	0	100	0	100	0	100
	0	100	0	100	0	90	10	...[3]	...[3]
	10	100	0	100	0	50	50	100	0
34° F	20	35	65	50	50	10	90	0	100
	30	0	100	0	100	0	100	0	100
	40–80	0	100	0	100	0	100	0	100

[1] Catalase positive cocci and rods.
[2] Catalase negative, mostly rods.
[3] Plates overgrown with catalase positive, spreading colonies of aerobic spore formers.

Since catalase negative organisms were the only ones to grow appreciably during storage of the products, about 10% of the isolates were chosen for further study. This was done by selecting at random at least one culture from each product at each storage temperature for each of five or more sampling periods. In this way a total of 50 isolates were chosen. These were distributed approximately equally among the 4 products and between the 2 temperatures.

All of the organisms were Gram positive rods except one, a tetracoccus. None reduced nitrate to nitrite, and none grew well, if at all, on nutrient agar slants incubated aerobically. None grew at 45° C, but all grew at 15°, at 30°, and 37° C. All were facultative with respect to oxygen and only two grew at pH 9.6.

On the basis of the additional tests made, it was possible to assign the organisms to the following 3 groups:

1. *Hetrofermentative rods* (14 cultures): Acid and gas from glucose and sucrose; no acid from lactose or xylose; most cultures failed to produce acid from arabinose, mannitol or sorbitol; salt tolerance ranged from 6.5 to 10.0%; all but 3 cultures produced greening of wieners.

2. *Homofermentative organisms*

   a. Acid produced from lactose (6 rods): Acid but no gas from glucose; none of the cultures fermented xylose, mannitol or sorbitol; four of the six fermented sucrose, and two fermented arabinose; salt tolerance ranged from 7.5 to 9.5%; most cultures caused greening of wieners.

   b. *Lactose not fermented* (29 rods, 1 tetracoccus): Acid but no gas produced from glucose and sucrose; no acid from xylose, mannitol and sorbitol and only four cultures of the 30 fermented arabinose; salt tolerance ranged from 8.5 to 10.0%; 18 of the 30 cultures caused greening of wieners.

As can be seen in Table 2, no group was restricted to a single product or temperature. The smallest group, the lactose fermenting homofermentative rods, were obtained from 2 products and at both temperatures whereas representatives from the other 2 groups were isolated from all products at both temperatures.

TABLE 2

Incidence of the different groups of isolates in the four processed meat products

	Number of cultures							
	Bologna		Pressed ham		Smokie patties		P & P loaf	
	45° F	34° F	45° F	34° F	45° F	34° F	45° F	34° F
Heterofermentative	2	2	2	2	1	1	3	1
Homofermentative								
Lactose fermenters			2	1			3	
Nonlactose fermenters	5	4	3[1]	1	3	6	3	5

[1] One culture was a tetracoccus.

Attempts to assign the isolates to known species of lactic acid bacteria generally were unsuccessful. Part of the heterofermentative rods have been shown to be *Lactobacillus viridescens (8)*, but most of the others differed in several important characteristics from recognized species of lactobacilli. A more complete taxonomic appraisal of the isolates is in progress.

Certain characteristics of the isolates as a group have practical interest. All fermented glucose and most fermented sucrose, whereas few attacked the other carbohydrates tested. About one-third of them produced gas from glucose and two-thirds caused greening of wieners. All grew in broth containing 6.5% salt and almost all tolerated at least 8.5%. Two-thirds of the cultures grew even in the presence of 9.5 to 10.0% salt.

The heat resistance of the cultures varied widely. Of 11 cultures tested in APT broth, the thermal death times at 140° F (60° C) ranged from 1.25 min to 28 min. High (or low) thermal death times were not associated with organisms from any one product.

DISCUSSION

The brine concentration as calculated from the salt and water content of the meats did not change during the time of storage. This is in agreement with the work of Steinke and Foster *(11)* who found that Saran has a very low permeability to water vapor and that it can be used successfully to maintain the original appearance of the product. The brine concentrations in the 4 meats were as follows: P & P Loaf—4.2%, Smokie Patties—4.6%, Bologna—5.0%, and Pressed Ham—5.8%. As can be seen in Figure 1, the 4 meats spoiled in approximately the same order as they increased

in brine concentration. Smokie Patties, however, deteriorated slightly faster than P & P Loaf. The surfaces of the patties were quite rough; therefore, a large number of spaces existed between them, providing additional surfaces for microbial growth and slime production. The other 3 meats differed in this respect, since they were sliced from large loaves, and each slice in the package was in complete contact with its neighbors. The formation of the slime between the Smokie Patties can be likened to the slime formation in the small spaces between Visking casing and Pliofilm overwrap on bologna and liver sausage, as reported by Steinke and Foster *(11)*.

All of the lactic acid bacteria characterized were shown to tolerate at least 6.5% salt. The highest brine concentration found in the 4 meats studied was well below this (Pressed Ham—5.8%). Even though the same types of organisms were found in the ham as in the other meats (Table 2), the growth rate in this product was slower (Figure 1) than in the others; consequently, a longer period was required for the lactics to establish themselves as the predominant flora (Table 1). It seems, therefore, that even if the organisms can tolerate the concentration of salt present in a processed meat, their growth rate will decrease as the brine concentration increases. The salt concentration required to inhibit the growth of all these organisms would render the meat unpalatable. Spoilage, therefore, could not be prevented by salting alone, although a slight increase in the salt content of the products other than ham might delay their spoilage considerably.

Spoilage of cold meats by lactic acid bacteria, as described by Niven and co-workers *(6, 7)*, consisted of the production of green discolorations and slime. Oxygen is required for the formation of the green color, and, since the meat studied in this work was under vacuum, no discolorations were noted in the samples. However, most of the organisms causing the spoilage were capable of causing such a discoloration when inoculated onto wieners. This property, then, is not peculiar to the heterofermentative species, *L. viridescens*, of Niven's group *(6, 7, 8)*.

Spoilage of the meats in this study was definitely retarded by the lower storage temperature (Figure 1). Adequate refrigeration, therefore, cannot be ignored if a maximum storage life is desired for the products. Even at the lower temperature, however, the lactics were able to reach vast numbers in time.

In his discussion of green sausage discolorations Niven *(5)* concludes that control of the causative organisms depends on the rigid practice of good sanitation. This is especially important with products such as those studied here. After the loaves of the meats are processed, they must undergo further handling during slicing and packaging. The organisms eliminated during the previous processing could be replaced by fresh contaminants, both from workers and from the slicing and packaging machines, if rigid sanitation were not practiced.

Nothing found in the work reported here would suggest a modification of Niven's *(5)* conclusions. Under practical conditions the meat packer cannot control the types of organisms in his products, but he can control the number of organisms. Both salt concentration and low temperature have practical limits and can help control the growth of spoilage organisms to a certain extent. Plant sanitation, however, determines the number of organisms present in the finished product. If the number is excessive, none

of the preservation methods can inhibit the bacteria sufficiently to assure the maximum storage life of the product. Plant sanitation, therefore, cannot be overemphasized.

SUMMARY

The spoilage of four types of vacuum packed, sliced cold meats during storage at refrigerator temperatures was shown to consist of slime formation and development of atypical flavors. Putrefaction did not occur.

The products had significantly longer storage lives at 34° F (1.1° C) than at 45° F (7.2° C).

Lactic acid bacteria were found in great numbers. The growth of these bacteria seemed to be the cause of the spoilage, since no other types of organisms could be isolated from the high dilutions required to determine the microbial content of the meat.

The thermal death-time studies showed that the heat tolerance varied greatly among the isolates.

Although these bacteria could tolerate high concentrations of salt, a small additional quantity might further inhibit them and lengthen the storage life of the products.

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ANTHOCYANINS. I. THE INFLUENCE OF STRAWBERRY AND GRAPE ANTHOCYANINS ON THE GROWTH OF CERTAIN BACTERIA[a]

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Few studies have been reported dealing with the influence of anthocyanins on the growth of bacteria. Blank and Suter *(3)* investigated the effect of anthocyanins on pathogenic bacteria and reported that concentrations of 0.02 and 0.1M had no bactericidal action. The work of Blank and Suter was substantiated by Mandrik *(7)* who also reported that the anthocyanins had no apparent effect on pathogenic bacteria.

Masquelier and Jensen *(9, 10)*, in studying the bactericidal action of red wine, reported that pigments isolated from grapes to be used in red wine were not bactericidal. On hydrolysis, a fraction containing partially demethoxylated oenidol had definite bactericidal activity. Masquelier and Jensen found that as wine ages oenoside (malvidin 3-monoglucoside) was slowly converted to oenidol (malvidin). The oenidol thus formed possessed a phenol coefficient of 33. Masquelier and Jensen thought this might explain the bactericidal action of red wine.

Zimmerman *(17)* found that strawberry and grape anthocyanins significantly influenced the growth rates of *Lactobacillus acidophilus* and Cameron's Putrefactive Anaerobe No 3679 (P.A. 3679). Zimmerman found that the growth rate of P.A. 3679 was stimulated by anthocyanins. The growth of *L. acidophilus* was stimulated by 0.008 to 0.025 mg % pigment while 2.5 mg % depressed the growth rate. He found no difference in the effect of strawberry and grape anthocyanins.

EXPERIMENTAL PROCEDURE

Isolation of anthocyanins

The anthocyanins investigated in the present study were isolated from commercially frozen strawberries and commercially frozen and bottled Concord grape juice. Frozen strawberries were macerated in a Waring blender and the pulp removed from the juice by pressing through three layers of cheese cloth.

Extraction: The pigments were extracted from the fruit juice with acidified (1% HCl) 1-butanol. Two liters of the acidified butanol were used per liter of fruit juice. The aqueous and organic phases were separated and extraction of the pigments repeated. The pigments were precipitated into an aqueous phase by the addition of petroleum ether to the pigment-butanol solution. The organic phase was discarded.

Chromatographic solvents: Two solvents were prepared by thoroughly shaking 1-butanol, acetic acid, and water (4:1:5 v/v) in a separatory funnel. On standing the mixture separated into 2 phases. Herein, the upper phase (water-saturated butanol) is referred to as solvent A and the lower phase (butanol-saturated water) as solvent B *(14)*.

Chromatographic separation: The aqueous pigment extracts were concentrated *in vacuo* and heavily streaked on rectangular (8 x 12 in) Whatman No 1 filter paper. Using solvent A and the ascending chromatographic technique the chromatograms were de-

[a] Contribution from the College Experiment Station, University of Georgia, Athens, Georgia. Approved as Journal Paper No 101.

veloped for 16 hr. The bands were cut from the chromatograms and eluted with acidified (1%HCl) methanol. This solution was evaporated to dryness *in vacuo*, the solids redissolved in water, and again streaked on the filter paper. The ascending chromatographic procedure was repeated using solvent B. The bands were cut from the chromatograms and eluted with acidified methanol. The pigment-methanol solution was concentrated *in vacuo* and placed in a desiccator to allow the pigment to crystallize. The crystals were dissolved in water and the aqueous solution sterilized by passing through a Seitz filter.

Bacterial cultures

Cameron's Putrefactive Anaerobe No. 3679 (P. A. 3679): Spore suspensions of P. A. 3679 were prepared on pork-extract broth by the procedure of the National Canners Association *(12)*. The spores were suspended in physiological saline and stored at 3° C prior to use.

Escherichia coli: A culture of *E. coli* was maintained on nutrient agar slants (Difco B 1). Twelve hours prior to use the culture was inoculated into nutrient broth (Difco B 3).

Lactobacillus acidophilus: A culture of *L. acidophilus* was maintained on litmus milk (Difco B 107). Twelve hours prior to use sub-cultures were inoculated into litmus milk.

Media for bacterial growth

The medium used to study the effects of anthocyanins on P.A. 3679 was pork-extract broth with added dehydrated pork. The growth media for *E. coli* and *L. acidophilus* were, respectively, nutrient broth (with and without added glucose) and micro inoculum broth (Difco B 320). The tubes of P.A. 3679 were overlaid with petroleum jelly and placed in a sealed glass container in which oxygen was removed by a burning candle. All tubes contained 10 ml of medium into which had been added various concentrations of sterile anthocyanin pigments at levels from 0.1 to 50.0 mg %.

Bacterial growth measurements

P. A. 3679: The culture tubes were incubated at 37° C for 12 days and bacterial counts were made daily. Counts were made by the modified deep-agar shake procedure of Miller, Garrett, and Prickett *(11)*. The medium used for the count was thioglycolate agar (Difco B 363 with 0.5% dextrose added).

E. coli: The growth of the *E. coli* cultures was estimated initially by plate counts and later by turbidimetric measurements at 550 mmc. A Bausch and Lomb "Spectronic 20" Spectrophotometer-Colorimeter was used for turbidimetric measurements. The tubes were incubated in a water bath at 30° C and turbidimetric measurements were made at 30-min intervals for 30 hr. Conventional plate counts were initially used in measuring the growth of *E. coli*, but to permit measurements to be made at closer intervals, turbidimetric measurements were later used.

L. acidophilus: Growth of *L. acidophilus* was estimated by direct plate counts on micro-inoculum agar overlaid with non-nutrient agar.

RESULTS AND DISCUSSION

Two anthocyanins were isolated from strawberries. The primary pigment of strawberries has been identified as pelargonidin 3-monoglucoside *(15)*. Spectral absorbance measurements, using a Beckman Spectrophotometer (Model DU), indicated that the major pigment isolated from strawberry was the same as pelargonidin 3-monoglucoside reported by Sondheimer and Kertesz *(15, 16)* and Markakis, Livingston, and Fellers *(8)*. When the major pigment of strawberries was isolated and chromatographed, an R_f value of 0.42 was found in a solvent of 1-butanol–acetic acid-water (4:1:1 v/v). The aglucone obtained by acid hydrolysis had R_f values of 0.78 and 0.68 in 1-butanol-2N HCl solvent and acetic acid-HCl-water solvent, respectively. Bate-Smith *(1)* found the R_f values to be 0.80 and 0.68 in the same solvent systems.

The second pigment of strawberries has been identified by Lukton, Chichester, and Mackinney *(6)* as cyanidin 3-monoglucoside. The minor pigment isolated in this study is apparently the same as the pigment found by Lukton *et al. (6)*. The R_f value of this pigment was found to be 0.31 in the 1-butanol–acetic acid-water solvent. Lukton *et al. (6)*, Bate-Smith and Westfall *(2)*, and Parkinson *(13)* reported the R_f value of cyanidin 3-monoglucoside, in butanol–acetic acid-water to be 0.32, 0.33, and 0.30, respectively. The aglucone of the minor pigment in this study had R_f values of 0.69, 0.46, and 0.25 in 1-butanol-2 N HCl, acetic acid-HCl-water, and 1-butanol-acetic acid-water solvents, respectively. The R_f values reported by Bate-Smith *(1)* and Joslyn and Peterson *(5)* in the same solvent systems were 0.69, 0.50, and 0.24.

Two of the major pigments of grapes have been identified as malvidin 3-monoglucoside and delphinidin 3-monoglucoside *(14)*. The chromatographic separations of grape pigments in solvent A yielded 4 and sometimes 5 distinct bands. Chromatographic identifications indicated that delphinidin 3-monoglucoside predominated. The R_f values of the anthocyanidin (aglucone) of this pigment were 0.33 and 0.29 in 1-butanol-2 N HCl solvent and acetic acid-HCl-water solvent, respectively. Bate-Smith *(1)* found the R_f values to be 0.35 and 0.30 in the two solvent systems.

As may be seen from Figures 1 and 2, the growth of P.A. 3679 was greatly influenced by both pelargonidin 3-monoglucoside and delphinidin 3-monoglucoside. Figures 1 and 2 also show that the effects of pelargonidin 3-monoglucoside and delphinidin 3-monoglucoside on the germination of spores and growth of P.A. 3679 were nearly identical. Both anthocyanins stimulated the growth of P.A. 3679 in that the lag phase of growth was reduced. The re-

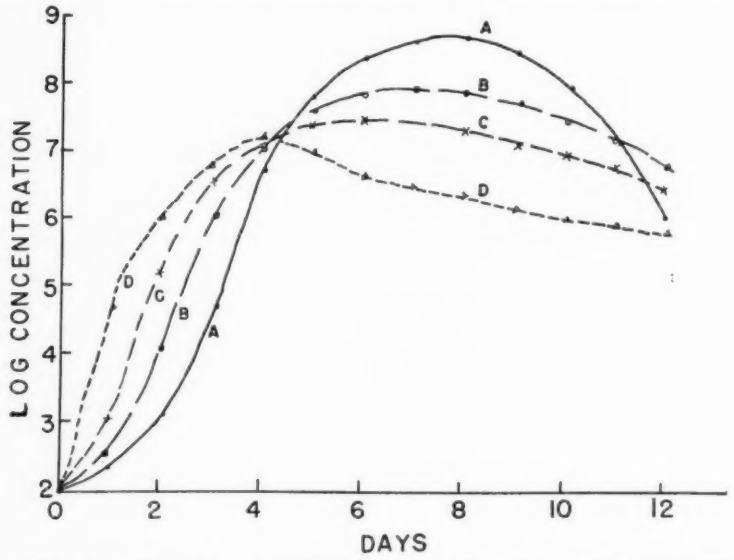

Figure 1. Influence of pelargonidin 3-monoglucoside on the growth of P.A. 3679 in pork extract media. A—control; B—0.5 mg %; C—5.0 mg %; D—50.0 mg %.

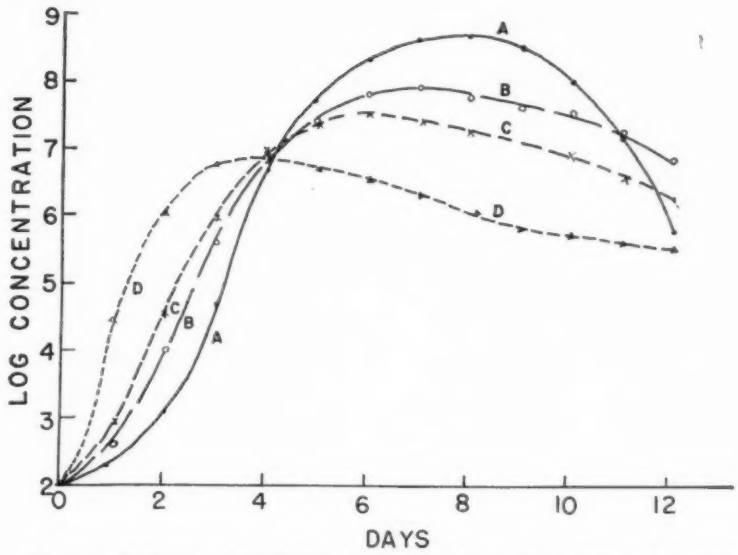

Figure 2. Influence of delphinidin 3-monoglucoside on the growth of P.A. 3679 in pork extract media. A—control; B—0.5 mg %; C—5.0 mg %; D—50.0 mg %.

duction in the lag phase was directly proportional to the anthocyanin concentration. An inhibition was also found in that the maximum growth was inversely proportional to the concentration.

Masquelier and Jensen *(9, 10)* and Jensen *(4)* reported that malvidin, but not its glucoside, had definite bactericidal activity. This might explain why the inhibition in maximum growth was noted. During the incubation of the cultures the anthocyanins may have undergone hydrolysis to form their respective aglucones.

Figure 3 shows the growth curves of *E. coli* in nutrient broth with added pelargonidin 3-monoglucoside and cyanidin 3-monoglucoside. The concentration of the anthocyanin in the media for which the curves are presented was 10 mg %. Concentrations of 0.1 mg % and 1.0 mg % had no noticeable effect on the growth of *E. coli* as measured turbidimetrically.

The authors felt that glucose might be split by hydrolysis from the pigment and be responsible for the growth stimulation. Therefore, nutrient broth with 1.0% glucose added was also used as a growth medium for *E. coli*. The results of the determinations, with 10 mg % pigment added, are shown in Figure 4. Again concentrations of 0.1 mg % and 1.0 mg % of anthocyanin had no noticeable effect on the growth of *E. coli*.

The growth of *E. coli* was stimulated by pelargonidin 3-monoglucoside and cyanidin 3-monoglucoside in media with and without added glucose. Stimulation was greater in the medium containing no added glucose. This might indicate that glucose present as the glucoside may serve as a carbon source for the bacteria. However, this glucose probably was not solely responsible for the stimulation since the concentration of added glucose was

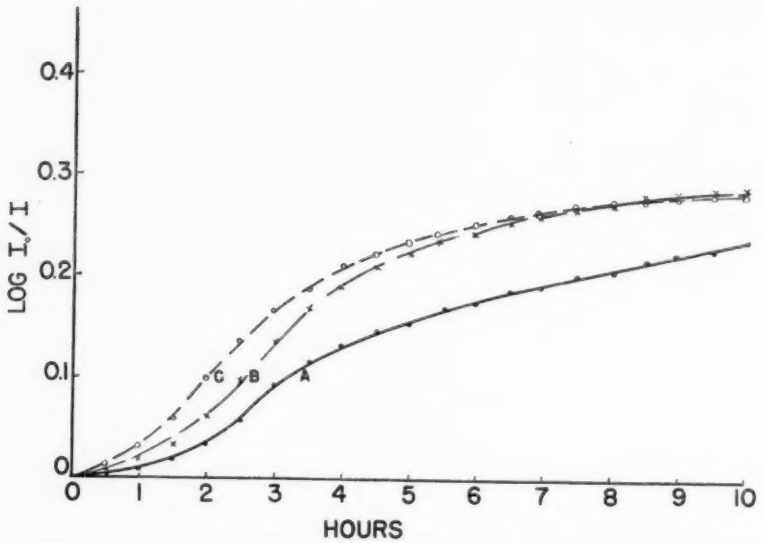

Figure 3. Influence of cyanidin 3-monoglucoside and pelargonidin 3-monoglucoside on the growth of E. coli in nutrient broth. A—control; B—10 mg % cyanidin 3-monoglucoside; C—10 mg % pelargonidin 3-monoglucoside.

approximately 250 times that of the natural glucose present in the 10 mg % pigment solution. (This assumes that all pigment was present as the monoglucoside.)

At 28 hr of growth, the turbidity readings of *E. coli* cultures in the media containing no added glucose were approximately the same whether or not pigments were present. The medium containing added glucose gave similar results. However, a great difference existed between the media with no added glucose and those containing added glucose. Up to about 28 hr of incubation, the turbidity was greater in media containing glucose; beyond that point, the turbidity of the media without added glucose exceeded the turbidity of the media containing glucose.

An inhibition in the maximum growth of *L. acidophilus* was consistently noted when pelargonidin 3-monoglucoside and delphinidin 3-monoglucoside were added to the medium. However, plate counts were not made at sufficiently frequent intervals to establish whether or not the pigments affected the lag phase. For media containing either of the two anthocyanins in concentrations of 50 mg % the maximum growth was approximately 70% that of the control.

Results of the present study are supported in part by the findings of Zimmerman *(17)*. Zimmerman found that the growth rates of P.A. 3679 were increased and the maximum growth of *L. acidophilus* cultures was inhibited by strawberry and grape anthocyanins, both on solid and liquid media. He also found that these pigments had no apparent effect on the growth of *E. coli*. Differences in the findings of this study and the findings of Zimmer-

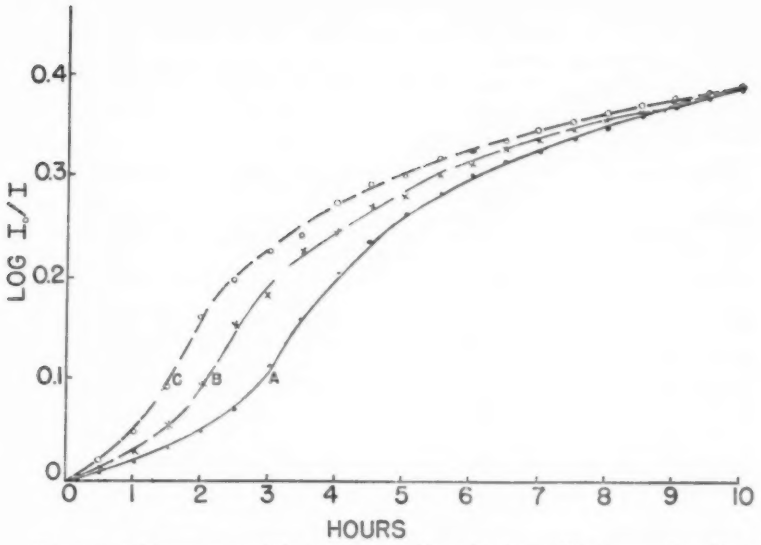

Figure 4. Influence of cyanidin 3-monoglucoside and pelargonidin 3-monoglucoside on the growth of E. coli in nutrient broth (with 1.0% added glucose). A—control; B—10 mg % cyanidin 3-monoglucoside; C—10 mg % pelargonidin 3-monoglucoside.

man might be explained by the differences in procedures of the two studies. Zimmerman may not have made counts at sufficiently frequent intervals to obtain a complete picture of the effect of the pigments on the bacteria. He also separated extracted pigments as groups by means of an alumina adsorption column and the results he reported were not made on anthocyanins fractionated into individual pigments.

SUMMARY

Strawberry and grape anthocyanins, identified as pelargonidin 3-monoglucoside, cyanidin 3-monoglucoside, and delphinidin 3-monoglucoside, influenced the growth of *E. coli* and *L. acidophilus*.

Similar results were obtained when spores of P.A. 3679 were germinated and grown in the presence of pelargonidin 3-monoglucoside and delphinidin 3-monoglucoside. Initially these pigments accelerated growth by reducing the lag phase of growth directly proportional to the anthocyanin concentration. An inhibition was also noted in that the maximum growth was inversely proportional to the pigment concentration.

Concentrations of 10 mg % pelargonidin 3-monoglucoside and cyanidin 3-monoglucoside accelerated the growth of *E. coli*. However, after 28 hours of growth turbidimetric readings for media with and without the pigments were nearly the same.

An inhibition was found in the maximum growth of *L. acidophilus* in the presence of both pelargonidin 3-monoglucoside and delphinidin 3-monoglucoside. The maximum growth for *L. acidophilus* in anthocyanin-fortified media (50 mg per 100 ml) was approximately 70% of the growth in the unfortified media.

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THE EFFECT OF STORAGE ON ASCORBIC ACID CONTENT OF GAMMA RADIATED POTATOES[a]

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The effect of ionizing radiations on the vitamin C content of foods is known to depend upon the level of radiations. Nickerson *et al. (7)* showed that high-voltage cathode rays of 2000 krep (1860 krad) caused 64–92% loss of this vitamin in asparagus, broccoli, green beans, and spinach. Proctor and O'Meara *(8)* also demonstrated that high-voltage cathode radiations caused some loss of ascorbic acid in solutions, and to a lesser extent, in orange juice. Brownell and associates *(2)* maintained that even radiations of 10 krep (9.3 krad) reduced the ascorbic acid content of potatoes, and that the effects were more pronounced as the dosage level increased, but no data were given. On the other hand, Mickaelsen and Roer *(6)* reported that the vitamin C content was decreased in both radiated and non-radiated potatoes during the first seven months of storage at approximately 40° F, but was restored after this period, and that ascorbic acid levels of the radiated samples were higher than those of the controls.

In the light of the present divergent views regarding the effect of storage on the ascorbic acid content of potatoes which had been subjected to ionizing radiations to inhibit sprouting, there appeared to be a need for further study. Cloutier *et al. (3)* reported the effect of storage on the carbohydrate content of two varieties of potatoes treated with gamma radiation. This paper reports data on the ascorbic acid content of the same set of samples.

MATERIAL AND METHOD

Two varieties, Katahdin and Netted Gem (Russet Burbank) were used. Details of experimental design, gamma radiation treatments, and storage conditions were described by Cloutier *et al. (3)*.

Tubers were cut in quarters lengthwise. Each sample was a quarter of the same tuber which was used in the determinations of carbohydrates. Five replicates were analyzed for each treatment and for each storage period of each variety of potatoes, except for those samples remaining after 9 months of storage. In this case because of the relatively low variation between replicates previously obtained, only two replicates of 2½ quarters of potatoes were used.

Samples were blended in 20% meta-phosphoric acid with cracked ice in a Waring blender, and the blends diluted with distilled water to contain 2% meta-phosphoric acid. The filtrates were analyzed immediately for vitamin C. The vitamin C was determined

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[a] This work forms part of a co-operative project which was set up in 1956 between the Canada Department of Agriculture, the Atomic Energy Company of Canada and the Department of National Health and Welfare to study the effect of gamma radiation on the storage and culinary properties, ascorbic acid, reducing sugars, sucrose and starch content of two commercially important varieties of potatoes grown in Canada: the Katahdin and Netted Gem (Russet Burbank) varieties. The experimental design and results of this study were presented at the 15th International Horticulture Congress in Nice, France, April 1958. Acknowledgment is made to the committee in charge of this project for permission to publish these results.

in the form of reduced ascorbic acid by the 2,6-dichlorophenolindophenol titration technique *(1)*.

RESULTS AND DISCUSSION

The results of the experiment are summarized in Table 1 and the statistical tests of significance are shown in Table 2. No values were available for control potatoes after 9 months storage at 68° F because these potatoes were decayed and discarded. Storage temperature appeared to be the most important factor influencing vitamin C content. For both varieties, vitamin C

TABLE 1

Effect of storage on mean ascorbic acid content of potatoes after treatment at different levels of gamma radiation

Storage		No of replicates analyzed	Ascorbic acid (mg/100 g fresh wt)[2]			
Months	Temperature		Control	7.9 krad	10.2 krad	14.0 krad
	°F					
		Katahdin Variety				
¼	52–54	5	17.60	16.82	15.28	16.86
4½	40	5	7.93	7.34	7.17	7.09
9	40	2	7.95	7.13	7.77	7.45
4½	68	5	10.70	10.90	10.86	11.17
9	68	2	discarded	11.08	10.75	11.13
		Netted Gem Variety				
¼	52–54	5	13.06	12.50	11.92	11.70
4½	40	5	7.98	7.72	7.73	7.35
9	40	2	8.27	7.70	7.62	8.04
4½	68	5	11.56	12.65	12.54	13.34
9	68	2	discarded	11.90	12.31	11.82
4½	Recond.[1]	5	10.89	10.60	10.74	10.32
9	Recond.[1]	2	10.24	11.20	11.05	10.94

[1] Reconditioned: potatoes removed from 40° F storage to 68° F for 3 weeks.
[2] Fresh weight at time of sampling; mean weight loss due to storage less than 5%.

content was preserved much better at 68° F than at 40° F. Highly significant differences between treatments, *i.e.* level of radiation at each temperature, were apparent at 4½ months but disappeared at 9 months storage. There were consistently significant differences between varieties within temperatures. Katahdin potatoes had higher initial ascorbic acid content but after storage contained significantly less ascorbic acid. In both varieties, the greatest drop in ascorbic acid content occurred during the first 4½ months storage. This loss was in agreement with the results of Schreiber and Highlands *(9)* which were published after this study was undertaken. At 9 months, however, in contrast to their data, the ascorbic acid content remained at a low level and the incidence of rotting was zero. It is possible that the higher temperatures during their commercial storage increased both the ascorbic acid content and incidence of rotting.

The effect of "reconditioning" the potatoes was shown in the case of samples of Netted Gem variety which were transferred from 40° F to 68° F and kept at the higher temperature for three weeks reconditioning before ascorbic acid determinations were made. As a result, the amounts of vitamin C rose from about 8 to 11 mg per 100 g, approaching the level of those stored

TABLE 2

Analysis of variance of ascorbic acid content of gamma radiated potatoes

Source of variation	No storage		4½ Months storage		9 Months storage	
	df	Mean square	df	Mean square	df	Mean square
Between storage temps			1	345.6032[2]	1	95.5457[2]
Between treatments within temp	3	6.4456	6	1.5345[2]	5	0.2042
Between varieties within temp	1	147.8403[1]	2	13.5273[2]	2	1.5051[2]
T x V within temp	3	3.9776	6	0.4242	5	0.1874
Error	32	2.8440	63	0.3721	13	0.2076
Total	39		78		26	

[1] Significant at P = .05.
[2] Significant at P = .01.

at 68° F for the full time. A similar effect was observed by Leichsenring and associates *(4)*, who obtained slight increases of ascorbic acid after a similar reconditioning of potatoes. The increase observed in the present work was an effect of storage temperature and not radiation.

The most likely explanation for the apparent increase of vitamin C as a result of reconditioning appears to be either that the vitamin was derived from dehydroascorbic acid or from some other precursor present in the tuber. It is possible that part of the original reduced ascorbic acid was oxidized to dehydroascorbic acid during storage. In the course of reconditioning, the latter was converted to the original reduced ascorbic acid, thus showing an apparent increase in the vitamin content. These changes were similar to those described by McCombs *(5)*, who showed that in some vegetables there were marked increases of reduced ascorbic acid after harvest, and at the same time there were decreases of dehydroascorbic acid content.

SUMMARY

Levels of gamma radiation did not cause consistent or large variation in the ascorbic acid content of potatoes. The main cause of loss of ascorbic acid was storage. Storage at 40° F resulted in lower ascorbic acid values than storage at 68° F. Low ascorbic acid content caused by low temperature storage could be partially restored by reconditioning, suggesting that some of the ascorbic acid had been reversibly oxidized.

No marked differences in the loss of ascorbic acid were observed between samples stored for 4½ months and for 9 months.

Acknowledgment

The authors are indebted to Miss Constance E. Cox for the statistical analysis and to J. A. Campbell for assistance in preparing the manuscript.

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STUDIES ON THE PURIFICATION OF TOMATO POLYGALACTURONASE

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In recent years, various investigators have demonstrated the complex nature of pectic enzymes *(cf. 2)*. Excluding enzymes preferentially attacking pectins with a high degree of esterification, there are two well-established pectic enzymes which attack polygalacturonic acid. Endo-polygalacturonase of yeast, which is a single enzyme *(14)*, hydrolyzes pectic acid randomly to a mixture of galacturonic and digalacturonic acids. Exopolygalacturonase, a component of the pectic enzyme complex of certain fungi, removes single galacturonic acid units from the end of a pectic acid polymer, ultimately hydrolyzing it completely to galacturonic acid. Aside from the observation of McColloch *(9)*, based on electrophoretic studies of a tomato extract, that the material contained two or possibly three components having polygalacturonase activity, there are no studies indicating the possible complexity of tomato polygalacturonase.

The present knowledge of the properties of tomato polygalacturonase *(8, 9, 11)* is based on the use of relatively crude enzyme preparations. Since nearly the total pectic enzyme content of tomatoes is associated with the pulp *(4)*, most investigators used extraction with salt as a means of solubilizing the enzymes. This procedure solubilizes the pectinesterase (PE) as well as the polygalacturonase (PG). The latter is present in much lower concentration than the former *(8, 10)*. McColloch *(9)* extracted macerated tomatoes with NaCl and dialyzed the extract against distilled water, causing precipitation of the pectic enzymes. The precipitate was redissolved in 10% NaCl and dialyzed again. This process was repeated three more times, each time using less salt solution. The final precipitate was dissolved in 0.5M sodium acetate solution adjusted to pH 7.0. Pithawala *et al. (16)* have reported that under these conditions PE was considerably inactivated, but they gave no quantitative results about PG. They were able to remove a considerable amount of inert protein by adding 20% (w/v) of $(NH_4)_2SO_4$. Other recent investigators *(8, 11)* prepared tomato PG by extracting it with NaCl from tomatoes, saturating the extract to 90% saturation with $(NH_4)_2SO_4$ and taking up the precipitated enzyme in sodium chloride solution or in acetate buffer.

The purpose of the present publication is to describe methods by which a more thorough purification of tomato PG can be achieved, resulting in a water soluble preparation. The properties of the purified enzyme are described in a following publication *(13)*.

MATERIALS AND METHODS

Determination of reaction rates. Polygalacturonase activity was estimated by following the liberation of reducing groups from a 0.5% solution (dry weight basis) of Wichmann pectic acid *(12)* at pH 5.0 and 30° C by a modified Willstätter-Schudel hypoiodite procedure *(3)*. Activity was expressed in polygalacturonase units per ml of enzyme

solution or per mg protein (PGu/ml or PGu/mg P). One unit of PG activity is that amount of enzyme which liberates one millimole of aldehyde groups per minute. Since the purpose of this investigation was a purification of polygalacturonase, pectinesterase (PE) was only determined qualitatively or semi-quantitatively. This was done by observing the amount and nature of calcium pectinate formed when a 1% pectin solution (pH 7.5), containing 0.2M NaCl, after treatment with certain enzyme fractions, was supplied with a calcium chloride solution. An approximate estimate of the removal of PE during purification of the PG can be obtained if the conditions of the test are standardized. The crude enzyme produced a solid gel within a few minutes, after addition to the pectin solution. The purified fractions were diluted to a PG concentration similar to that in the starting material, before adding the enzyme to the test solution.

Determination of protein concentration. The method of Lowry *et al. (7)* was used. The protein concentrations were read from a standard curve, previously constructed with crystallized bovine plasma albumin (Armour and Company, Chicago, Illinois). Standards were freshly prepared with each series of determinations.

Preparation of adsorbents. Pectic acid gel was prepared according to the procedure of Phaff and Demain *(14)* and stored at 5° C with toluene as preservative. The pH of the gel was 3.4. Calcium pectate gel was prepared according to the procedure described by Newbold and Joslyn *(12)*. The gel obtained from 100 ml of 1% pectin solution was mixed with 1 l of distilled water and stored at 5° C. Its pH was 4.7. Calcium phosphate gel was prepared according to the procedure of Kunitz *(5)*. It had a pH of 8.0.

RESULTS AND DISCUSSION

Extraction of tomato PG. Since the PG in tomatoes is present in very low concentration, initial steps were directed to obtain the crude enzyme in more concentrated form. Advantage was taken of the fact that tomato PG is associated with the pulp. About 150 lb of firm ripe Pearson tomatoes were cut into large pieces and pressed in a hydraulic rack and cloth press. The juice was discarded. The pulp was resuspended in water and pressed again. Washing was repeated once again, finally yielding about 25 lb of pressed pulp. This step undoubtedly removes a significant part of the water soluble proteins of the fruit.

About 5% by weight of NaCl was mixed thoroughly with the pulp together with 2 l of 5% NaCl solution. After standing overnight with toluene as a preservative, the mixture was pressed and the salt extract filtered through "Hyflo" supercel (Johns-Manville). About 9.5 l of clear filtrate were obtained. The solution was stored at 5° C with 0.01% merthiolate as preservative. This prevented bacterial spoilage during subsequent dialysis, a trouble which could not be controlled by adding toluene or chloroform. Merthiolate has no influence on the action of tomato PG. The activity of this crude extract was 1.8×10^{-3} PGu/ml and the specific activity was 1.29×10^{-3} PGu/mg protein. The extract did not lose any activity during storage for 12 months at 5° C.

Removal of NaCl by dialysis. The finding of McColloch *(9)* that upon dialysis of the NaCl-extract against distilled water tomato PG precipitates together with a large amount of inert protein was confirmed. Dialysis against 0.1M acetate buffer pH 4.5 also resulted in precipitation of PG and other proteins. It was found, however, that if the dialysis was done against tap water (a fairly hard water), followed by a short period of dialysis against distilled water, most of the enzyme remained in solution. About 250 ml portions of the salt extract were poured into Visking bags and suspended in a tall glass cylinder. Tap water was introduced at the bottom and allowed to

overflow. After 16 hours the chloride content of the ingoing water was about the same as that of the outgoing water. The glass cylinder was then filled with distilled water, which was replaced by fresh distilled water after 2 hours. During the entire period the bags were agitated frequently. Two hours after the final dialysis against distilled water a sample was taken. Only a small amount of precipitate had formed and about 86% of the PG activity was in soluble form. The pH of the dialyzed solution was 6.2. The small amounts of salt remaining did not interfere in the following adsorption experiment. There was no inactivation of the tomato PG during the dialysis procedure.

Adsorption on pectic acid gel. Since Phaff and Demain *(14)* successfully used pectic acid gel at 4° C as an adsorbent for concentrating yeast polygalacturonase, this method was applied first for concentrating and purifying tomato PG. Using this procedure, the surprising observation was made that the more

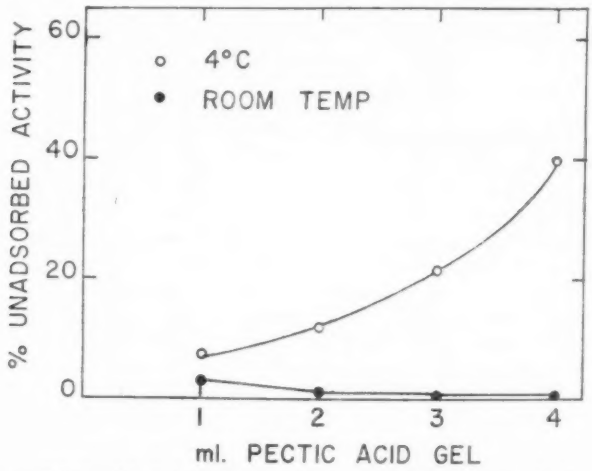

Figure 1. Relationship between concentration of pectic acid gel and the amount of tomato PG adsorbed at 4° C and at room temperature. Volume of enzyme was 20 ml in all cases.

gel was added to the dialysate the less PG was adsorbed (Fig. 1). This behavior was contrary to that of yeast endo-PG *(14)*. It was observed, however, that in the supernatant liquid, containing the unadsorbed PG-activity, a fine gel-like precipitate formed when it was allowed to stand at room temperature for several hours. A considerable part of the PG activity was precipitated in this gel. In view of this observation the adsorption procedure was repeated at room temperature with various amounts of gel added to the dialysate. As shown in Figure 1, nearly all the activity could be removed from the solution. The reason for the effect of temperature on adsorption is not clear. To determine the purification and concentration obtainable by this method, a relatively large scale experiment was done using 600 ml dialysate (pH 7.8) and 60 ml of pectic acid gel (final pH 4.35). After stirring for 5 min (longer periods did not result in increased adsorption) the gel was cen-

trifuged and washed with 75 ml of 0.01 N acetic acid (pH 3.5) for 15 minutes. After centrifuging, the adsorbed protein was eluted with 30 ml 1 M acetate buffer pH 5.0 by stirring for 15 min, followed by centrifugation. The purification and concentration are presented in Table 1. All activities were determined after dialysis against 1M acetate buffer pH 4.5 by the hypoiodite procedure. The enzyme was concentrated 14-fold and the increase in specific activity shows a removal of about 30% of inert protein.

TABLE 1

Changes occurring during concentration of tomato PG by pectic acid gel

Sample	Volume	Activity	Total activity	Percent activity	Protein concentration	Specific activity
	ml	*PGu/ml x 10^3*	*PGu*		*mg/ml*	*PGu/mg P x 10^3*
Crude dialysate	600	1.39	0.834	100	1.078	1.29
Supernatant after adsorption	650	0	0	0	0.090	0
Washing	75	0	0	0	0.028	0
Eluate	42	16.65	0.70	84	9.02	1.85

Dialysis of concentrated tomato PG. When the enzyme solution in 1 M acetate buffer was dialyzed against distilled water or 0.1 N acetate buffer pH 4.5, a precipitate formed which could be redissolved by increasing the buffer strength to one molar. The results in Table 2 show that only 8.7% of the activity remained in the supernatant liquid after dialysis against 0.1 N acetate buffer pH 4.5. The precipitate was washed twice with 10 ml 0.1 M acetate buffer pH 4.5. The 2.3 fold increase in specific activity shows that about 57% of inert proteins was removed by this treatment. When this procedure was repeated, all the PG activity precipitated, but only a small additional increase in specific activity was obtained. In spite of a fairly good purification, the problem of obtaining a water soluble tomato PG still remained.

Ammonium sulfate fractionation. Pithawala *et al. (16)* have reported that a considerable amount of protein is precipitated from tomato extracts in phosphate buffer pH 8.0 at 20% concentration (w/v) of $(NH_4)_2SO_4$. Thus,

TABLE 2

Changes occurring during the dialysis of concentrated tomato PG (pectic acid gel eluate) against 0.1 M acetate buffer pH 4.5

Sample	Volume	Activity	Total activity	Percent activity	Protein concentration	Specific activity
	ml	*PGu/ml x 10^3*	*PGu*		*mg/ml*	*PGu/mg P x 10^3*
Dialyzed eluate[1] before centrifugation	18	16.65	0.300	100	9.02	1.85
Supernatant after centrifugation	18	1.46	0.0263	8.7	3.8	0.384
Precipitate dissolved in 10 ml 1 M acetate buffer pH 4.5	10	24.2	0.242	80.7	5.61	4.32
Enzyme again dialyzed vs 0.1 M Ac pH 4.5, centrifuged and precipitate redissolved in 1 M acetate buffer pH 4.5		25.6			5.34	4.78

[1] Enzyme used as suspension.

a fractionation with $(NH_4)_2SO_4$ was attempted using the concentrated, purified tomato PG described in the previous section. Increasing amounts of $(NH_4)_2SO_4$ were added to the protein solution in 1 M acetate buffer pH 5.0 at 0° C. The precipitated protein was centrifuged at high speed, washed with 0.1 M acetate buffer of the same $(NH_4)_2SO_4$ concentration, and again centrifuged. The washings were discarded. The washed protein fractions were dissolved in 5 ml of 1 M acetate buffer pH 4.5 and dialyzed against 0.1 M acetate buffer before measuring their activity and protein concentrations. The results are presented in Table 3. The activity not accounted for was probably lost in the washings. When the protein obtained between 0 and 0.5 saturation was dialyzed, some precipitate formed. However, the fraction precipitating between 0.5 and 0.7 saturation (62.4% of the total activity; specific activity 7.04×10^{-3} PGu/mg protein) remained in solution during dialysis against dilute buffer or distilled water. A qualitative test for pectinesterase indicated that most of this enzyme precipitated in the first fraction and only a weak activity was found in the second fraction. The over-all recovery in the three-step procedure was 42%.

TABLE 3

Changes occurring during ammonium sulfate fractionation of concentrated tomato PG. All precipitates were dissolved in 5 ml 1 M acetate buffer pH 4.5

Saturation	Activity	Recovery	Protein concentration	Specific activity
	PGu/ml x 10^3	*%*	*mg P/ml*	*PGu/mg P x 10^3*
0	24.2	100	5.61	4.32
0 –0.5	2.33	9.6	1.68	1.39
0.5–0.7	15.1	62.4	2.145	7.04
0.7–1.0	0	0	0.081	0

Since a water soluble preparation was obtained by the above procedure, a direct $(NH_4)_2SO_4$ fractionation of the crude NaCl extract was tried, but a soluble preparation was not secured. Ammonium sulfate fractionation of the pectic acid gel eluate resulted in a soluble tomato-PG, but its specific activity was lower than that obtained by the three-step procedure.

Purification of water soluble tomato-PG with other adsorbents. Preliminary studies were done with the water soluble enzyme described in the previous section (three-step purification procedure). Calcium phosphate gel, calcium pectate gel and pectic acid gel were used. It was found that the purified enzyme was very poorly adsorbed on pectic acid gel, whereas adsorption on the other two gels was complete. Since the eluate from calcium phosphate gel had the highest specific activity and the most favorable PG/PE ratio, more detailed studies were done with this gel. Adsorption on calcium phosphate gel is done at pH 8.0. The stability of tomato PG at this pH value was surprising, since polygalacturonases with an optimum pH of about 4.5 from other sources have been reported to be very sensitive when exposed to high pH values *(15)*. Since calcium phosphate gel adsorbed tomato PG selectively, leaving most of the PE in the supernatant liquid, it was of interest to determine if the same procedure could be applied directly to the dialyzed crude tomato extract, thus eliminating several steps. A sample of tomato extract

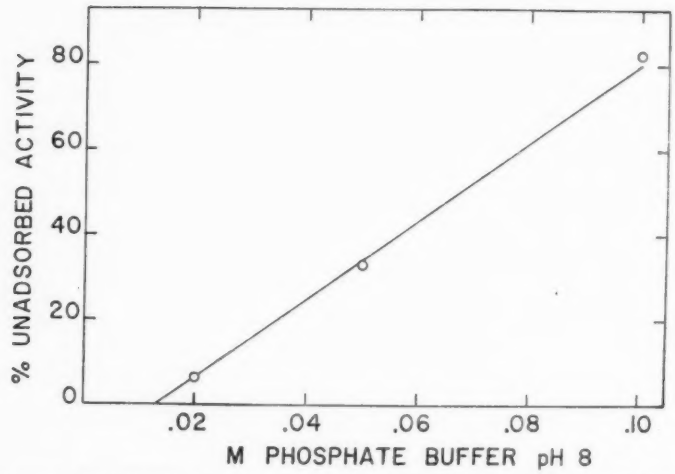

Figure 2. Relationship between the amount of tomato PG adsorbed on calcium phosphate gel (10% volume by volume) and the strength of the phosphate buffer (pH 8.0).

was first dialyzed against tap water, followed by distilled water causing precipitation described earlier. It was found, however, that the precipitate readily dissolved by adding phosphate buffer at pH 8.0. In order to determine the optimum conditions for adsorption two series of tests were made. In the first series 10% calcium phosphate gel was added in all cases and the buffer strength was varied. After mixing for 5 min, followed by centrifugation, the

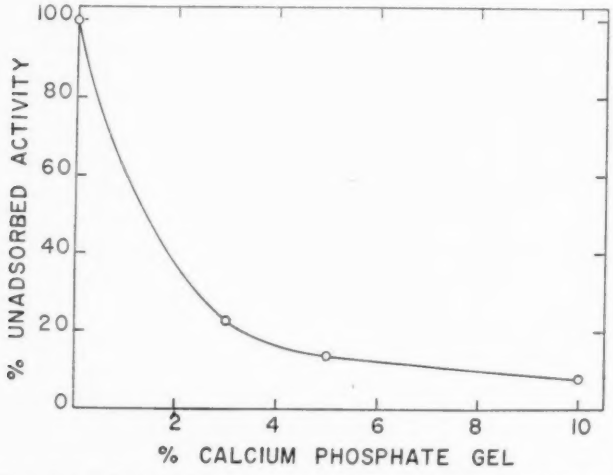

Figure 3. Relationship between the concentration of calcium phosphate gel and the amount of tomato PG adsorbed. Solvent 0.02M phosphate buffer pH 8.0.

supernatant liquids were dialyzed against 0.1 M acetate buffer pH 4.5. No precipitation occurred. The unadsorbed activities are shown in Figure 2. On the basis of this experiment 0.02 M phosphate buffer was chosen for the adsorption. In the second series the amount of calcium phosphate gel was varied and the buffer concentration was kept constant. The results are shown in Figure 3. Addition of 5% gel was chosen as a satisfactory concentration for adsorption. The PG adsorbed in these exploratory tests was easily recovered by treating the gel with 1 M acetate buffer pH 4.5 in which most of the gel dissolved. Dialysis against dilute acetate buffer or water did not cause precipitation of the enzyme after this purification procedure. A larger scale experiment was done next in order to determine recoveries and specific activities. The results are shown in Table 4. The original solution consisted of

TABLE 4

Purification of tomato PG with calcium phosphate gel adsorption at room temperature

Sample	Volume	Activity	Recovery	Protein concentration	Specific activity
	ml	*PGu/ml x 10^3*	*%*	*mg P/ml*	*PGu/mg P x 10^3*
Original	288	1.43	100	0.802	1.78
Supernatant after adsorption	305	0.238	17.6	0.424	0.56
A[1] 1 M acetate eluate I	12.5	9.17	55.7	2.08	4.41
1 M acetate eluate II	13.0	2.89	18.2	0.576	5.02
1 M acetate eluate III	12.8	0.794	4.9	0.176	4.51
B[1] 0.1 M phosphate eluate I	11.5	4.17	23.3	0.924	4.51
0.1 M phosphate eluate II	11.5	4.33	24.2	0.777	5.58
1 M acetate eluate III	12.0	3.34	19.4	0.756	4.42

[1] After adsorption, the accumulated gel was divided into two equal parts for the purpose of elution.

288 ml of crude dialysate. Because of the large volume it was impractical to remove all the salt and no appreciable precipitate formed during the dialysis. To the solution were added 12 ml 0.5 M phosphate buffer pH 8.0 so that the final molarity became 0.02 with respect to phosphate. The mixture was centrifuged and the small amount of precipitate, which did not contain any PG activity, was discarded. The clear supernatant liquid (300 ml) was stirred for 5 minutes with 15 ml of phosphate gel. After centrifugation, the accumulated gel was washed 3 times by stirring with 30 ml 0.02 M phosphate buffer pH 8.0. The washings were discarded and the gel divided into two equal parts. The adsorbed protein from one part of the gel was eluated with 3 successive 10 ml portions of 1 M acetate buffer pH 4.5. The second part of gel was eluated with two 10 ml portions of 0.1 M phosphate buffer pH 8.0 followed by a third elution with 10 ml of 1 M acetate buffer pH 4.5. The entire adsorption and elution procedure was done at room temperature. All activities were determined, after dialysis against 0.1 M acetate buffer pH 4.5, by the hypoiodite procedure. The adsorption was not as efficient as in the preliminary small scale experiments, probably because the dialysate was not free of chloride. It would probably be better to do the adsorption from 0.01 instead of 0.02 M phosphate buffer when large volumes must be dialyzed.

The specific activities of the two series of eluates were quite similar, but much better recoveries were obtained with acetate buffer alone than with

phosphate followed by acetate buffer (*79% versus 67%*). In addition the eluates with Na-acetate remained clear upon dialysis against dilute buffer, whereas the phosphate eluates formed some precipitate under these conditions and contained more PE. The procedure for obtaining a concentrated, partially purified, water soluble tomato PG was greatly simplified by the use of the calcium phosphate gel adsorption and elution procedure, since only one step is involved. The specific activity of the phosphate gel eluate was somewhat lower than that of the enzyme obtained by the three-step procedure. However, when the phosphate gel eluate was fractionated by $(NH_4)_2SO_4$ (fraction precipitating between 0.5 and 0.9 saturation) the specific activity rose to 5.8 x 10^{-3} PGu/mg protein. Since in the three-step procedure the specific activity did not always reach 7.0 x 10^{-3} PGu/mg protein when various batches were prepared (range 6.0 to 7.0 x 10^{-3} unit), the calcium phosphate procedure, followed by $(NH_4)_2SO_4$ fractionation, must be considered the recommended one at present.

Purification on a mixed bed of ion-exchange resins. Since Anyas-Weisz *(1)* showed that it was possible to remove pectin esterase from a fungal preparation of polygalacturonase with complete recovery of the PG activity, this procedure was tried with the tomato enzyme. The two resins used were Dowex 50 x 4% (200–400 mesh) in the hydrogen form and Dowex 2 (50–100 mesh) in the hydroxyl form. The two resins were mixed thoroughly in equal amounts (volume by volume) and a small column containing 10 ml of the mixed resin was set up. Five ml of an aqueous solution of tomato PG (the eluate from pectic acid gel, precipitated with $(NH_4)_2SO_4$ between 0.5 and 0.9 saturation) was passed slowly over the column and the effluent was collected in fractions of 10, 5 and 5 ml each. The results in Table 5 show that only 46% of the PG activity was recovered, although the specific activity of the second fraction (13% recovery) had increased about two-fold. In another small scale experiment in which tomato PG of higher specific activity was used (Table 5) the PG recovery was only about 20%. In view of the low

TABLE 5

Changes occurring in tomato PG during treament with a mixed-bed of cation and anion exchange resins

Sample	Volume	Activity	Recovery	Protein concentration	Specific activity
	ml	*PGu/ml x 10^3*	*%*	*mg P/ml*	*PGu/mg P x 10^3*
Original	5	6.39	100	2.112	3.03
Fraction 1	10	1.068	33.4	0.274	3.9
Fraction 2	5	0.835	13.0	0.141	5.92
Fraction 3	5	0	0	0	0
Original	2	6.04	100	0.858	7.04
Fraction 1	10	0.238	19.7	.....	.....
Fraction 2	5	0	0	.....	.....

recoveries of PG this method was not explored further as a method of purifying the PG. It is possible that the uneven particle sizes of the two resins may have caused difficulties in preparing a homogeneous mixture of the two resins.

Comparison with other pectic enzymes. The purest preparation of tomato

PG (7.0×10^{-3} PGu/mg protein) had a specific activity which was 5.5 times as high as that of the crude salt extract, corresponding to a removal of about 82% of foreign proteins. The specific activity of this preparation is considerably lower than that reported for yeast polygalacturonase *(14)*, 0.163 PGu/mg protein, or that of a partially purified preparation of fungal origin *(6)*, which contained 0.272 PGu/mg protein (converted from 1.7 PGu per mg total nitrogen). It is not known at the present time whether this low activity is a characteristic of tomato PG or whether it is due to insufficient purification.

SUMMARY

Two procedures have been described for the purification of tomato polygalacturonase. The starting material was a salt extract of thoroughly washed, hydraulically pressed, fresh tomato pulp. The first procedure involved removal of most of the salt by dialysis, adsorption on pectic acid gel at room temperature and elution with 1 M acetate buffer pH 5.0. The enzyme precipitated when the solution was dialyzed against 0.1 M acetate buffer pH 4.5 causing further purification. The precipitate was dissolved in 1 M acetate buffer and the fraction precipitated by $(NH_4)_2SO_4$ between 0.5 and 0.9 saturation constituted the purified water soluble enzyme. The second procedure consisted in adsorbing the dialyzed salt extract on calcium phosphate gel at pH 8.0 and treating the gel with 1 molar acetate buffer at pH 4.5. This single step procedure resulted in a water soluble preparation. The specific activity could be increased by fractionating the eluate with $(NH_4)_2SO_4$ (0.5–0.9 saturation). In the purest preparation obtained, about 82% inert protein was removed during purification, corresponding to an increase in specific activity from 1.29×10^{-3} PGu/mg protein to 7×10^{-3} PGu/mg protein.

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PROPERTIES OF PURIFIED TOMATO POLYGALACTURONASE

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In a previous paper *(17)* methods have been described for the purification of tomato polygalacturonase (PG). The PG in a crude salt extract of washed tomato pulp was purified 5.5 times, corresponding to an increase in specific activity from 1.29×10^{-3} PGu/mg protein to 7×10^{-3} PGu/mg protein. Most of the pectinesterase was removed during purification. In contrast to the crude enzyme the purified enzyme was soluble in water and in dilute buffers. It has been shown that crude preparations of tomato PG cause a random type of hydrolysis of pectic acid to D-galacturonic acid *(8, 12)*. If the rate of hydrolysis of pectic acid is taken as 100, the rates of hydrolysis of tetra-, tri-, and digalacturonic acids are 7.0, 1.58 and 1.05, respectively *(8)*. The purpose of the present paper is to give more detailed information on the mechanism of hydrolysis of pectic acid and of certain oligouronides by purified tomato PG. In addition oxidized and reduced derivatives of the oligouronides were used as substrates in order to define the enzyme action more precisely. Our results have been described briefly in a preliminary note *(15)*.

EXPERIMENTAL PROCEDURES

Tomato PG. Purified enzyme solutions were prepared by methods described in detail in the preceding paper *(17)*.

Preparation of substrates. Acid-soluble pectic acid of low molecular weight was prepared by the procedure of McCready and Seegmiller *(11)*. Its average degree of polymerization (DP) was found to be about 14 (ratio of carboxyl groups/aldehyde groups). Di- and trigalacturonic acids were obtained by the procedure of Phaff and Luh *(18)*, and tetragalacturonic acid by the method of Demain and Phaff *(3)*. Reduced oligouronides, in which the terminal D-galacturonic acid unit is changed to L-galactonolactone, were prepared by the procedure of McCready and Seegmiller *(11)*. Oxidized oligouronides, in which the end D-galacturonic acid molecule is changed to mucic acid lactone, were made by hypoiodite oxidation of the aldehyde group of the oligouronides as described by Patel and Phaff *(16)*. Wichmann pectic acid was prepared by the procedure of Newbold and Joslyn *(14)*.

Designation of bonds. In all instances, the glycosidic linkage next to the terminal reducing group (or its modification) will be termed bond 1. The next bond is 2 and so on.

Paper chromatography. The method of Jermyn and Isherwood *(6)* as modified by Demain and Phaff *(3)*, was used to determine the movement of oligogalacturonides in relation to that of D-galacturonic acid.

Protein determination. Protein was determined colorimetrically by the method of Lowry *et al. (7)* using crystallized bovine plasma albumin as the standard.

Determination of reaction rates. Glycosidic hydrolysis of Wichmann pectic acid (0.5%) was followed by the hypoiodite procedure at pH 5.0 and at 30° C as described in the preceding paper *(17)*. A polygalacturonase unit (PGu) is that amount of enzyme which will release 1 mmole of aldehyde groups per minute from pectic acid under the above conditions. With pectic acid (DP 14) and in some experiments with oligouronides a semimicro modification of the hypoiodite procedure was used as described by Patel and Phaff *(16)*. For measuring the rate of hydrolysis of oligouronides at different pH values the colorimetric method of Somogyi *(21)* was used. The optimum heating time in a boiling water bath of the alkaline copper reagent was found to be 15 minutes and there was

no evidence for breakdown of oligouronides during this period. Aliquots of 0.05 ml reaction mixture were taken with a micropipette and added to the reaction tubes containing 1.95 ml distilled water and 2 ml of the Somogyi reagent. After heating for 15 min, the tubes were cooled in an ice bath and 1 ml of Nelson's *(13)* arsenomolybdate reagent was added to develop the color. The solution was diluted to 25 ml and the optical density was measured with a green filter (No 54) in a Klett-Summerson photoelectric colorimeter. The optical density was proportional to the galacturonic acid monohydrate content in the range of 0–350 μg. The μmoles reducing groups calculated with known quantities of oligouronides agreed very well with the experimental values based on galacturonic acid.

RESULTS

Optimum pH for the hydrolysis of oligouronides and pectic acid. Since Demain and Phaff *(2)* showed that the optimum pH for the hydrolysis of tetragalacturonic acid by yeast endo-PG (a single enzyme) was between 3.3 and 3.5 and the optimum pH of pectic acid hydrolysis was 4.4–4.5, similar experiments were conducted with tomato PG. All hydrolysis rates reported by Luh *et al. (8)* had been determined at pH 4.5. The increase in reducing values was determined by the colorimetric procedure of Somogyi. The final buffer concentrations were 0.1 M acetate for pH 4.0, 4.5 and 5.0; 0.05 M phthalate for pH 3.5; single strength McIlvaine's buffer for pH 3.0, 5.4 and 6.0. The enzyme preparation had a specific activity of 4.4×10^{-3} PGu/mg protein. It was diluted in such a way that reasonably rapid reaction rates were obtained. Linear hydrolysis rates were found for the first 20% of the theoretical hydrolysis values of the dimer and the trimer, and for the first 50% in the case of tetragalacturonic acid. The results are presented in Figures 1, 2 and 3. It is evident that the hydrolyses of tri- and tetragalacturonic acid have optima approximately at pH 3.5 and at 4.5, whereas digalacturonic acid shows a single optimum at pH 4.4.

Because of the double peaks observed with tri- and tetragalacturonic acids, it was considered desirable to determine the activity of tomato PG on pectic acid in a range well below the reported optimum *(10)* at pH 4.5. Since the solubility of pectic acid of high molecular weight decreases rapidly at pH values below 4.0, acid soluble polygalacturonic acid (DP 14) was used as the substrate. The reaction mixture at pH 1.5 contained 0.05 M KCl and sufficient HCl was added to lower the pH to 1.5. pH values of 2.0 to 3.5 were maintained by 0.05 M phthalate buffer. The activities were determined by the hypoiodite procedure. The results shown in Figure 4 reveal that the hydrolysis of acid soluble pectic acid is characterized by two pH optima. The optimum at pH 4.5 confirms the value reported by McColloch and Kertesz *(10)*. In addition a second peak was found at pH 2.5. It would seem that the plateau observed between pH 3.0 and 4.0 might be due to an additive effect of the two maximal activities at pH 2.5 and 4.5. When the activity with an optimum pH of 2.5 was followed paper chromatographically with acid soluble pectic acid, a random splitting mechanism was evident.

Stability of tomato PG at 60° C. Because McColloch and Kertesz *(10)* showed that tomato PG contains a heat-stable component and since our experiments dealing with the optimum pH of tomato PG action indicated more than a single PG component, purified tomato PG was subjected to heat. It was considered possible that heat might selectively inactivate one of the postulated components of tomato PG. Another reason for doing this experiment was the finding by Brooks and Reid *(1)* that one of their PG preparations from *Aspergillus foetidus* contained an exo-PG component (end group splitting enzyme) which was very stable at 60° C and had an optimum pH with trigalacturonic acid at pH 3.0–3.5. Heating at 60° C for 20 min completely destroyed the endo-PG component (random splitting enzyme) of their preparation. The heat-stability of exo-PG was also demonstrated by Saito *(20)* who worked with a PG preparation from *Aspergillus niger*. The heat-stability of tomato PG was studied at 60° C, since it was found in preliminary tests that exposure of the enzyme to this temperature caused a rapid decrease in activity. Two ml samples of purified tomato PG (specific activity 5.8×10^{-5} PGu/mg P) in 0.1 M acetate buffer at pH 4.5 were heated in thin-walled tubes in a water bath and cooled in ice water. There was no precipitation of protein after heating. The results (Table 1) indicate that 91.6% of the PG activity was destroyed during the first 20 min. Heating for 30 min caused about the same amount of enzyme inactivation. When the enzyme, which was heated for 30 min, was allowed to act on pectic acid at pH 4.5 and the course

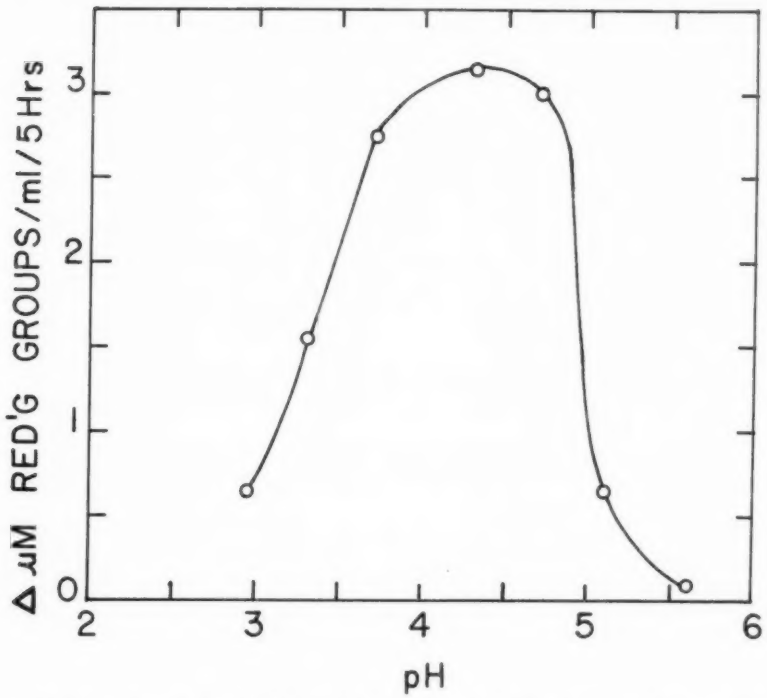

Figure 1. Hydrolysis of digalacturonic acid as a function of pH. Substrate concentration 0.5%. Enzyme concentration was 4.6×10^{-3} PGu per ml reaction mixture. Temperature 30° C.

of hydrolysis was followed by paper chromatography, random splitting of the chain to D-galacturonic acid was evident. The hydrolysis pattern was exactly the same as with an unheated enzyme solution adjusted to the same PG concentration as the heated preparation. Both hydrolysis patterns were similar to that reported by Luh, Leonard and Phaff *(8)*.

In a larger scale experiment 50 ml of the same purified tomato PG were heated for 30 min at 60° C and then fractionated at 4° C with ammonium sulfate as indicated in Table 2. The precipitated proteins were dissolved in 10 ml 0.1 M acetate buffer pH 4.5 and dialyzed against the same buffer until sulfate-free.

Although the PG before heat treatment was soluble in 0.5 saturated $(NH_4)_2SO_4$, after heating a considerable amount of heat-stable PG precipitated in half-saturated $(NH_4)_2SO_4$. This may be due to co-precipitation of active PG with denatured enzyme.

TABLE 1

Stability of purified tomato PG at 60° C in 0.1 M acetate buffer at pH 4.5

Heat treatment	Activity	Residual activity
	PGu per ml	%
None	8.9×10^{-3}	100
60° C for 20 minutes	0.75×10^{-3}	8.4
60° C for 30 minutes	0.725×10^{-3}	8.1

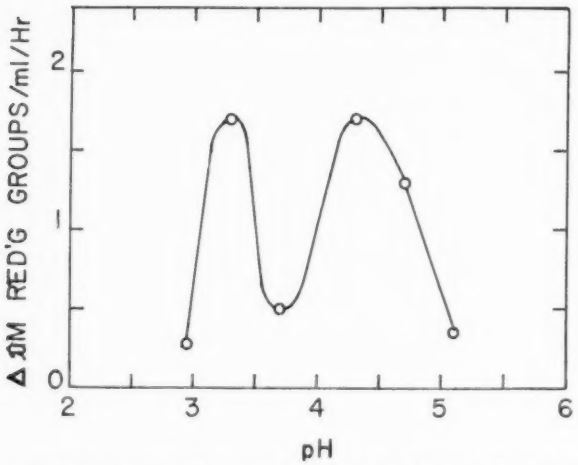

Figure 2. Hydrolysis of trigalacturonic acid as a function of pH. Substrate concentration 0.5%. Enzyme concentration was 1.8×10^{-3} PGu per ml reaction mixture. Temperature 30° C.

Since the original specific activity was 5.8×10^{-3} PGu per mg protein it can be seen from Table 2 that both fractions contained a large percentage of denatured protein. The lowering of the specific activity was of the same order of magnitude as the loss of about 92% in activity during heating (Table 1).

Since the hydrolysis of digalacturonic acid was characterized by a single optimum pH peak and is therefore presumably catalyzed by a single component of the tomato PG

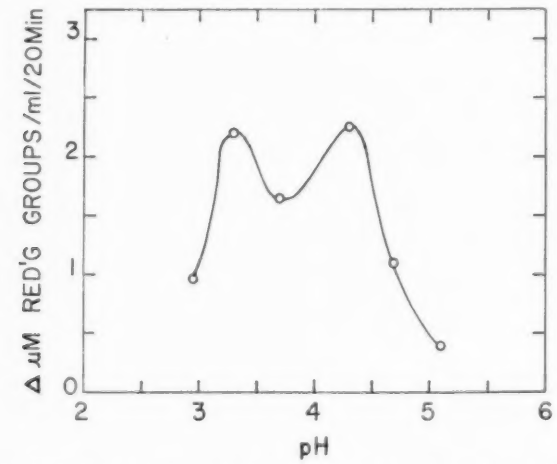

Figure 3. Hydrolysis of tetragalacturonic acid as a function of pH. Substrate concentration 0.5%. Enzyme concentration was 9.17×10^{-4} PGu per ml of reaction mixture. Temperature 30° C.

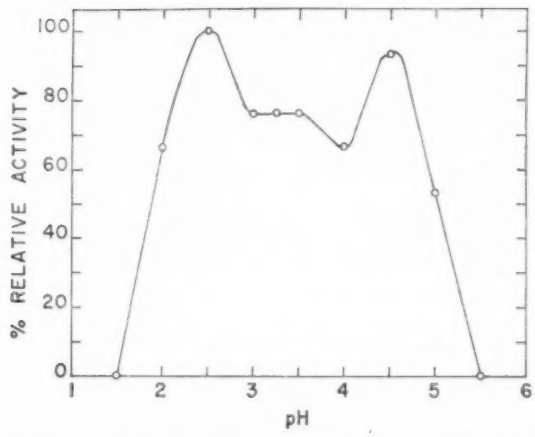

Figure 4. Relative rates of hydrolysis of acid soluble pectic acid (DP 14) as a function of pH. Substrate concentration 0.5%. Purified tomato PG concentration 2% (v/v).

complex, the hydrolysis of the dimer was followed paper chromatographically at pH 4.5 by the unheated enzyme solution and by the two heated fractions separated by $(NH_4)_2SO_4$. All 3 enzyme solutions were adjusted to 6.7×10^{-4} PGu per ml of reaction mixture. The results showed that digalacturonic acid was attacked by all 3 enzyme solutions at about the same rate and after 4 days no more dimer could be detected on the paper.

Thus the over-all results show that the heat-stable PG hydrolyzed pectic acid randomly and was able to break down digalacturonic acid at the same rate as the original solution before heating. No heat-stable exo-PG component could be detected.

Effect of pH on the stability of tomato PG. Some information regarding the stability of tomato PG at acid and alkaline pH values was desirable in order to compare it with PG components from other sources. Jansen and MacDonnell *(5)* found that after an initial destruction of fungal PG by holding the enzyme solution at pH 0.6 for 20 min, the remaining PG was quite resistant after further exposure to acid. Since the tomato PG was not inactivated at pH 8.0 during the calcium phosphate gel adsorption procedure *(17)*, the alkali treatment consisted of keeping the tomato PG at pH 10 for 15 min.

The enzyme solution used here was the same purified PG used for testing its heat resistance. It was dialyzed against distilled water to remove the acetate buffer. To a 2.5 ml aliquot of the enzyme solution, 3.4 N HCl was added slowly until the pH reached 0.6. After allowing it to stand at room temperature for 20 minutes, the pH was raised to 4.5 with 2.5 N NaOH, and the total volume made to 5 ml with distilled water. The exact time of the acid treatment was slightly more than 20 min, because lowering of the pH below 1.0 was rather time consuming. Similarly, a 2.5 ml portion of the enzyme solution was adjusted to pH 10 by adding 2.5 N NaOH. After allowing it to stand for 15 min, the solution was acidified with 3.4 N HCl to pH 4.5 and diluted to a total volume

TABLE 2

Fractionation of heat stable tomato PG with ammonium sulfate

Saturation	Volume	Activity	Protein concentration	Specific activity	Distribution
	ml	*PGu/ml x 10^3*	*mg P/ml*	*PGu/mg P*	%
0	50	0.725			100
0–0.5	13	1.67	3.84	0.43×10^{-3}	60
0.5–0.9	13	0.667	0.903	0.74×10^{-3}	24

TABLE 3

Stability of tomato PG at low and high pH values

Treatment	Volume	Activity	Stable PG
	ml	*PGu/ml*	%
None	2.5	7.5×10^{-3}	100
pH 0.6 for 20 minutes	5	0.5×10^{-3}	13.2
pH 10.0 for 15 minutes	5	2.5×10^{-3}	66

of 5 ml. During the adjustment of the pH with NaOH, the enzyme was momentarily exposed twice to a pH of 12 but was quickly brought down to pH 10.0. The activities of the treated enzyme were measured by the hypoiodite method with pectate as the substrate.

The results in Table 3 show that tomato PG is not very stable at low pH. In this respect, it resembles the behavior of the purified fungal PG of Jansen and MacDonnell *(5)* who found a residual activity of 9.3% after one treatment with acid. At pH 10, tomato PG was reasonably stable. The alkali inactivation of tomato PG could not be compared with the reversible inactivation by alkali of fungal PG as reported by Rahman and Joslyn *(19)* because of the lack of quantitative data by these authors. Further tests with the two treated preparations are described below (see Table 7).

Mechanism of hydrolysis of oligouronides and their derivatives

Tetragalacturonic acid. Luh, Leonard, and Phaff *(8)* have shown that at pH 4.5 the hydrolysis of tetragalacturonic acid by tomato PG produced trimer and monomer in the very early stages of the reaction. However, this preferred 3 + 1 split was not very clear cut. The situation is further complicated by the finding of two optimum pH values for tetramer hydrolysis. Since a concentrated, purified tomato PG was now available, a detailed chromatographic investigation on the hydrolysis of tetragalacturonic acid was carried out between pH 3.0–5.0. The purpose of this experiment was to find out if different mechanisms of attack on the tetramer might be demonstrated at pH values well removed from one or the other optimum. In this way interference of activities were expected to be minimized. The reaction mixtures consisted of 0.25% tetragalacturonic acid, 10% (v/v) tomato PG (9.17×10^{-4} PGu per ml reaction mixture) and appropriate buffers as were used in the section dealing with optimum pH values. The specific activity of the tomato PG was 4.4×10^{-3} PGu per mg protein. The hydrolysis products of the reaction mixtures were analyzed periodically by paper chromatography.

TABLE 4

Hydrolysis of tetragalacturonic acid by tomato PG at pH 3.0, 3.5, 4.5 and 5.0 as shown by paper chromatography

Time	Galacturonides															
	tetra-				tri-				di-				mono-			
pH	3.0	3.5	4.5	5.0	3.0	3.5	4.5	5.0	3.0	3.5	4.5	5.0	3.0	3.5	4.5	5.0
min																
0	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
6	+	+	+	+	+	+	+	+	(+)	(+)	(+)	(+)	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40	(+)	(+)	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+
60	−	−	−	(+)	+	+	+	+	+	+	+	+	+	+	+	+
hr																
2	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
5	−	−	−	−	(+)	−	−	(+)	+	+	+	+	+	+	+	+
24	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
168	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+

(+) represents weak spots.
Control experiments (no tomato PG) showed only tetragalacturonic acid at the end of the experiment.

The results in Table 4 show that at all pH values between 3.0 and 5.0, tetragalacturonic acid was hydrolyzed rapidly into tri-, di-, and monogalacturonic acids. During very early stages of the hydrolysis, there was a preferred 3 + 1 split of tetragalacturonic acid. However, trigalacturonic acid did not accumulate as is the case with endo-PG from yeast *(2)*. Even at pH 3.0 and 3.5, there was no definite evidence of a 3 + 1 split. The early appearance of digalacturonic acid can be explained best by the simultaneous splitting of bond *2* in tetragalacturonic acid, since the hydrolysis of the trimer formed is too slow to account for the rapid accumulation of the dimer.

Digalacturonic acid did not disappear at the end of 7 days when the reaction was carried out at pH 3.0 and 3.5. When the reaction was carried out at pH 4.5 and 5.0, digalacturonic acid was hydrolyzed completely at 7 days. These observations are in accord with the single optimum pH of 4.4 for the hydrolysis of digalacturonic acid. The rapid breakdown of the tetramer followed by the disappearance of the trimer over a wide pH range of 3.0–5.0 fits the existence of two optimum pH values for the hydrolysis of tri- and tetragalacturonic acids.

Derivatives of tetragalacturonic acid. The above studies did not give any indication whether bond 1, bond 3 or both can be attacked by tomato PG, in addition to bond *2*. This question was studied by subjecting reduced and oxidized tetragalacturonic acids to the action of purified tomato PG. If the numbering of glycosidic bonds is done from the modified end, then the hydrolysis of the first, second, and third bond would liberate tri-, di-, and monogalacturonic acids, respectively. The reaction was carried out both at pH 3.5 and at 4.5 because of the existence of the two optima. The experimental conditions were the same as those described in the previous section.

TABLE 5

Paper chromatographic analysis of galacturonides produced during hydrolysis of reduced and oxidized tetragalacturonic acid (TGA) at pH 3.5 and at 4.5. Enzyme concentration was 9.17 x 10^{-4} PGu per ml reaction mixture. Substrate concentration 0.25%. Temperature 30° C

Time	Reduced TGA pH 3.5			Reduced TGA pH 4.5			Oxidized TGA pH 3.5			Oxidized TGA pH 4.5		
	tri-	di-	mono-	tri-	di-	mono-	tri-	di-	mono-	tri-	di-	mono-
hr												
0	−	−	−	−	−	−	−	−	−	−	−	−
1	−	+	−	−	+	−	−	+	−	−	+	−
4	−	+	+	−	+	+	−	+	+	−	+	+
7	−	+	+	−	+	+	−	+	+	−	+	+
19	−	+	+	−	+	+	−	+	+	−	+	+
48	−	+	+	−	−	+	−	+	+	−	−	+
168	−	+	+	−	−	+	−	+	+	−	−	+

The results in Table 5 show that the reduced and oxidized modifications of tetragalacturonic acids were only attacked at bond *2* producing digalacturonic acid and a modified dimer as the initial hydrolysis products. The digalacturonic acid was then very slowly hydrolyzed to give galacturonic acid. Since already after 6 min tetragalacturonic acid produces a distinct spot for galacturonic acid (Table 4) it must be concluded that the galacturonic acid produced in this experiment could only have come from a secondary hydrolysis of the dimer formed. The hydrolysis patterns were the same at pH 3.5 and 4.5 for both modified substrates, except that the digalacturonic acid formed initially did not hydrolyze completely at pH 3.5. This confirms the single optimum pH of 4.4 for the dimer breakdown. The results also indicate that, at least initially, bond 1 in tetragalacturonic acid is protected from enzymatic hydrolysis by modifying the end group from aldehyde to a primary alcohol or to a carboxyl group. The hydrolysis rate of the third bond seems to be very slow or negligible since no galacturonic acid was produced at the start from the modified tetramers. The results indicate that in tetragalacturonic acid, the preferential hydrolysis of glycosidic bonds occurs from the reducing end of the chain. The hydrolysis at the first and second bond takes place quite rapidly

whereas bond 3 is probably not attacked at a significant rate. The comparative stability of bond 3 appears to be due to interference at the nonreducing end, which becomes progressively less as the bond in question is farther removed from that end of the molecule. In this connection Patel and Phaff *(16)* have shown that endo-PG from yeast cannot attack bond 3 in tetragalacturonic acid and bond 2 is hydrolyzed very slowly.

Trigalacturonic acid and its derivatives. Since the first bond of the modified tetramer was protected from enzymic hydrolysis by reducing or oxidizing the aldehyde group, it was of interest to find out if this generalization would also hold for trigalacturonic acid. In case the first bond is protected, the hydrolysis can occur only at a very slow rate at the remaining linkage (bond 2) and thus liberate galacturonic acid as the only reducing product. The experimental conditions were the same as those described in the previous section. The experiment was done at pH 4.5 only since the previous results have shown that the activities at pH 3.5 and 4.5 are very similar except for the hydrolysis of digalacturonic acid.

TABLE 6

Hydrolysis of trigalacturonic acid and its derivatives by tomato PG at pH 4.5 as shown by paper chromatography. Enzyme concentration was 9.17×10^{-4} PGu per ml reaction mixture. Substrate concentration 0.25%. Temperature 30° C

Time	Trigalacturonic acid			Reduced trigalacturonic acid		Oxidized trigalacturonic acid	
	tri-	di-	mono-	di-	mono-	di-	mono-
hr							
0	+	−	−	−	−	−	−
1	+	+	+	−	−	−	−
4	+	+	+	−	(+)	−	(+)
7	−	+	+	−	+	−	+
24	−	+	+	−	+	−	+
96	−	−	+	−	+	−	+
168	−	−	+	−	+	−	+

(+) represents weak spots.
Control experiments showed only the original substrates at the end of 7 days.

As expected, the results in Table 6 show that although trigalacturonic acid was hydrolyzed rapidly into dimer and monomer, its reduced and oxidized modifications were attacked only at a slow rate at bond 2 producing galacturonic acid. This confirms the indications obtained from the modified tetramer, that the first bond in such an oligouronide is protected from enzymic hydrolysis. A subsequent slow hydrolysis of D-galacturonosyl-mucic acid or D-galacturonosyl-L-galactonic acid cannot be detected by this method. The results also show that bond 2 in trigalacturonic acid is hydrolyzed very slowly in comparison to bond 1.

Digalacturonic acid and its derivatives. In order to make the study of oligouronide breakdown complete, the hydrolysis of digalacturonic acid and its modifications was carried out at pH 4.5 using the same experimental conditions as were used above. Digalacturonic acid was hydrolyzed completely within 4 days, but reduced and oxidized digalacturonic acids were not attacked even at the end of 14 days incubation at 30° C. Thus in the two previous experiments the modified dimers actually accumulate as true end products.

Ratio of activities on tetragalacturonic acid at pH 3.5 and 4.5. The finding of two pH optima for the hydrolysis of tri- and tetragalacturonic acids and only one optimum pH for the breakdown of digalacturonic acid was so far the only evidence which indicated the presence of more than one PG component. It was decided to follow the ratio of activities with tetragalacturonic acid at pH 3.5 and at 4.5 using a number of enzyme preparations treated in different ways. A significant change in this ratio would indicate the elimination of one of the postulated components of the tomato PG complex. Tetragalacturonic acid was chosen because of its more rapid rate of hydrolysis than trigalacturonic acid. Since the same substrate was used for determining the ratio, errors involved in

measuring the ratio would be smaller as compared to those encountered by using two different substrates such as pectic acid and digalacturonic acid.

The reaction mixture contained 20 mg tetragalacturonic acid (0.5% final concentration) dissolved in 1.6 ml distilled water and 2.0 ml of the appropriate buffer (0.1 M phthalate buffer pH 3.5 or 0.2 M acetate buffer pH 4.5). After the substrate and the enzyme solutions were brought to 30° C, 0.4 ml of the enzyme solution (10% v/v) was added and 1 ml samples were analyzed for increases in reducing groups with the semi-micro modification of the hypoiodite method.

TABLE 7

Hydrolysis of tetragalacturonic acid at pH 3.5 and 4.5 by tomato PG solutions treated in different ways

Treatment[1]	Activities *PGu/ml x 10⁴*		Ratio of activities
	pH 3.5 A	pH 4.5 B	A/B
1. None (concentrated PG kept in solution with 2% NaCl)	17.3	15.7	1.10
2. Water soluble preparation passed through a mixed bed of cation and anion exchange resins	1.14	0.919	1.24
3. I acetate eluate after phosphate gel adsorption	8.9	7.59	1.17
4. II acetate eluate	4.22	3.42	1.24
5. I acetate eluate further purified with $(NH_4)_2SO_4$	9.55	7.50	1.27
6. I phosphate eluate after phosphate gel adsorption	5.08	3.93	1.29
7. II phosphate eluate	5.23	4.22	1.24
8. Acetate eluate following phosphate eluate	5.23	4.07	1.28
9. Acid treatment—pH 0.6 for 20 min	0.514	0.455	1.13
10. Alkali treatment—pH 10.0 for 15 min	3.78	3.42	1.10
11. Heat stable PG precipitating between 0–0.5 saturation with $(NH_4)_2SO_4$	3.11	2.46	1.26
12. Heat stable PG precipitating between 0.5–0.9 saturation with $(NH_4)_2SO_4$	0.89	0.724	1.23

[1] See preceding paper.

The results are shown in Table 7. Although the enzyme solutions had undergone many different treatments, the ratio did not change significantly. Hence, no supporting evidence was obtained for the presence of more PG components than one. This probably indicates that if two PG components are present, their properties are similar and their separation by the methods used here does not look promising.

DISCUSSION

The existence of more than one kind of pectic glycosidase has been amply demonstrated in recent years. Demain and Phaff *(4)* have proposed a preliminary classification for the best known members of this group of enzymes. They considered the possibility that tomato PG might be a mixture of exo-PG (an end-group splitting enzyme) and endo-PG (a random splitting enzyme which cannot attack digalacturonic acid). A combination of these two enzymes would account for the complete hydrolysis of polyuronide to D-galacturonic acid. McCready *et al. (12)*, on the other hand, on the basis of their work, felt that there was no reason to assume the presence of more than one enzyme, although they did not exclude such a possibility. McColloch *(9)* subjected a salt extract of tomatoes to boundary electrophoresis and was able to demonstrate pectic acid hydrolyzing activity in samples representing two of the observed boundaries. One of the activities appeared to be considerably stronger than the other.

Our work reported here has given indications for the presence of at least two PG components. This conclusion was based on double pH optima with tri-, tetra- and acid soluble pectic acid and only a single optimum with digalacturonic acid. Since we did not effect a separation, the evidence is indirect. Although our preparations contained a heat-stable fraction, no evidence could be obtained that this fraction represented a heat-stable exo-PG, as has been demonstrated in fungi *(1, 20)*. With tetragalacturonic acid as the substrate at pH values well removed from one or the other optimum, no evidence was obtained for an end group-splitting enzyme. Thus no support for the hypothesis of Demain and Phaff was obtained in any of the experiments. The best way at present to differentiate tomato PG from mold PG and yeast endo-PG is by their action on digalacturonic acid and its reduced and oxidized derivatives. This is illustrated in Table 8. However, until the components of

TABLE 8

Comparison of polygalacturonases based on their ability to hydrolyze digalacturonic acid and its derivatives

Enzyme	Digalacturonic acid	Reduced digalacturonic acid	Oxidized digalacturonic acid
Yeast endo- PG[1]	−	−	−
Tomato PG	+	−	−
Mold PG[2]	+	+	not known
Exo-PG[3]	+	not known	+

[1] Patel and Phaff *(16)*.
[2] McCready and Seegmiller *(11)*.
[3] Brooks and Reid *(1)*.

tomato PG have been separated and purified it is difficult to speculate which activity is catalyzed by a particular component. It may be said that one activity is responsible for a complete random hydrolysis of pectic acid to D-galacturonic acid with an optimum pH of 4.4 to 4.5. However, there is no evidence that this chain of events is catalyzed by a single enzyme. On the basis of analogy with yeast endo-PG *(2)* there may be a second activity which hydrolyzes pectic acid randomly with an optimum pH of 4.5 and the oligouronides with an optimum at pH 3.4. This activity presumably stops at the digalacturonic acid stage since this acid showed only the optimum pH at 4.4. The optimum pH of 2.5 found for acid-soluble pectic acid is not comparable to any known pectic enzyme. It is possible that the enzyme representing this activity changes its optimal pH to 3.4 when the oligouronide stage is reached. A final classification and evaluation of the components of tomato PG must await physical separation of such components.

SUMMARY

Purified preparations of tomato polygalacturonase were used to study the mechanism and optimal conditions of the hydrolysis of polygalacturonic acid and of various oligogalacturonides. Both the hydrolysis of tetra- and of trigalacturonic acid was characterized by two pH optima at 4.5 and 3.5. Digalacturonic acid had a single optimum at pH 4.4. An acid-soluble pectic acid (average degree of polymerization 14) showed two optimum pH peaks at 4.5 and at 2.5. Paper chromatographic analysis of the reaction products

during the hydrolysis of oligouronides and their oxidized and reduced derivatives showed that in tetragalacturonic acid bond 1 (at the reducing end) is hydrolyzed rapidly, bond 2 slightly slower and bond 3 is not attacked at a significant rate. The pH had no effect on the patterns of hydrolysis. With trigalacturonic acid bond 1 is attacked rapidly, and bond 2 slowly. Digalacturonic acid is hydrolyzed slowly but not at low pH values. Exposure of the enzyme to heat, alkali or acid failed to change the pattern of pectic acid hydrolysis. Similarly the ratio of hydrolysis rates of tetragalacturonic acid at pH 3.5 and 4.5 failed to change as a result of various treatments of the enzyme preparation. Tomato PG probably contains at least 2 PG components, but their separation or differential inactivation was not accomplished.

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COMPATIBILITY OF PLANT, ANIMAL AND MICROBIOLOGICAL PROTEASES

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The growing acceptance of proteolytic enzymes in numerous industrial applications has expanded the scope of research in enzyme application to include those proteolytic enzymes not commercially available a few years ago. Meat tenderization is a striking example of an industrial application in which a great deal of attention has been given to an evaluation of the merits of a variety of proteases of plant, animal and microbiological origin *(2, 4, 6, 7, 8, 9)*. The work of Miyada and Tappel *(7)* of the University of California and Wang *et al. (9)* at the American Meat Institute Foundation demonstrated by chemical and histological methods, respectively, that proteases vary in their ability to attack the various components of muscle tissue. The apparent variation in specificity of these enzymes of different origin for muscle fibre, collagen, and elastin stimulated speculation regarding a combination of proteases which presumably could incorporate the desirable characteristics of each enzyme into one meat tenderizing formula thereby giving the tenderizer a broader spectrum of action on muscle tissue. Preliminary reference to the present work was made by one of the authors in a recent review of the basic research conducted in the field of enzymatic meat tenderization *(3)*. It has been observed for a number of years in this laboratory, that certain combinations of proteases of different origin were not compatible in solution. This was demonstrated by a loss in activity apparently through mutual digestion or inhibition. The significance of this finding was quite obvious since some meat tenderizers do incorporate more than one proteolytic enzyme in their formulas. Enzymatic chillproofing products used in the brewing industry contain, in some cases, a combination of proteases of different origin. The problem of compatibility exists here as it does in any other industrial application where proteolytic enzymes of different origin are used in combination.

The purpose of this work was to determine the extent of interaction, both inhibitory and synergistic, of various combinations of plant, animal, bacterial and fungal proteases under controlled experimental conditions.

EXPERIMENTAL

Almost all of the assay data were obtained with hemoglobin as the substrate. Since most of the enzyme research which led to this work was concerned with the tenderization of meat, the hemoglobin substrates used were buffered at the pH of meat (5.5). In those cases where maximum enzyme interaction occurred, gelatin was also used as a substrate to compare the extent of interaction found by hemoglobin assay.

Enzyme assay methods

Hemoglobin method. A modification of Anson's hemoglobin *(1)* method was used to evaluate proteolytic activity of pH 5.5. Accurately weigh 2.5 g of bovine hemoglobin enzyme substrate powder[a] and add 80 ml H_2O (grind into solution). Add 8 ml of

[a] Pentex Corp., Kankakee, Illinois

1 M–NaOH, 36 g urea and place in a 25° C water bath for 30–60 min to denature the hemoglobin. The substrate was prepared fresh each day.

Cysteine hydrochloride is used as an activating agent. Prior to use it is adjusted to a pH of 5.0.

Stock solutions of each enzyme are freshly prepared for each assay by dissolving 1 g of enzyme in boiled double distilled water to a volume of 200 ml. Aliquots are used from these stock solutions which give an optical density in such a range that if two enzymes are completely additive, the combination would be measurable with the same degree of precision as the individual enzyme solutions.

The assays are arranged in such a manner that the enzyme combination and each component of the combination could be evaluated each day. For example, when ficin and papain were combined, stock solutions of ficin and papain were prepared just prior to the one and one-half hour activation period. These were assayed individually along with the combination as follows:

Papain—5 ml stock solution per 200 ml in 0.1N cysteine HCl

Ficin—1 ml stock solution per 200 ml in 0.1N cysteine HCl

Papain and ficin—5 ml papain stock solution + 1 ml ficin stock solution per 200 ml in 0.1N cysteine HCl

The accurately weighed samples of enzyme are dissolved and made up to volume with boiled distilled water and cysteine hydrochloride so that the final volume is 0.1 molar with respect to cysteine hydrochloride. The enzyme activation period is 1½ hours and coincides with the interaction period for the enzyme combinations. Pipet 10 ml of hemoglobin substrate into a series of 50 ml test tubes and place in a 30° C water bath for 10–15 min. Two ml of the diluted enzyme solution are then added in various concentrations on the basis of protein content as determined by the Kjeldahl method. After 20 min, 20 ml of 5% trichloracetic acid are added rapidly to each test tube. The stoppered tubes are inverted twice and replaced in the 30° C bath for 30 min. After 30 min, each sample is filtered through S & S No. 589 Blue Ribbon filter paper. Absorbance of the filtrates is then measured at 280 mmc with a Beckman Spectrophotomteter Model DU. Blank determinations are carried out in the same manner except for the fact that the enzyme is added to 20 ml of trichloracetic acid before mixing with the hemoglobin substrate. A standard curve is then constructed for each enzyme by plotting absorbance of the filtrate vs. mg enzyme protein. All standard activity curves were linear over the range of enzyme concentrations used in this work (Fig. 1).

Gelatin liquefaction method. The viscometric technique used is a modification of the method as described by Lennox *(5)*. A 16% solution of Swift's Superclear Gelatin is prepared by placing 2690 ml of double distilled water in a four liter Pyrex beaker. Add 510 g of gelatin to the water with rapid stirring, dispersing it as evenly as possible. Allow to stand at room temperature for about 5 min or until it sets up and then place in a 90° C water bath; allow gelatin to stand for 10 min without stirring. Then agitate slowly for 35 min with a mechanical stirrer. At the end of 40 min, the temperature of the gelatin should be 80° C; stir for 5 min more; cool the gelatin to 50° C in a cold water bath and adjust the pH to 4.7 with 2N NaOH (the amount of NaOH required will vary with each lot of gelatin). At this point skim off any foam which may have developed during the agitation period. The gelatin should now be clear and free of air bubbles.

Weigh 250 g of gelatin solution into 300 ml tall form beakers and place in a water bath of 37.5° C for 15 min (until the gelatin reaches 37.5° C). The beakers should be covered with watch glasses. The level of water in the water bath must be slightly higher than the level of gelatin in the beakers. Two ml of diluted enzyme solutions are added to the gelatin and thoroughly mixed in with a glass rod for 1 min. The time of addition is noted and exactly 60 min later the viscosity of the gelatin is measured with a Brookfield Synchro-Lectric Viscometer spindle No. 1 at 60 rpm. Skim off any foam that may have formed on the gelatin before reading the viscosity. Three readings are taken; allow 8 revolutions before reading and average the 3 readings. Between sample readings, rinse the viscometer in hot water and then in a beaker of water at 37.5° C. Dry the spindle with a clean towel.

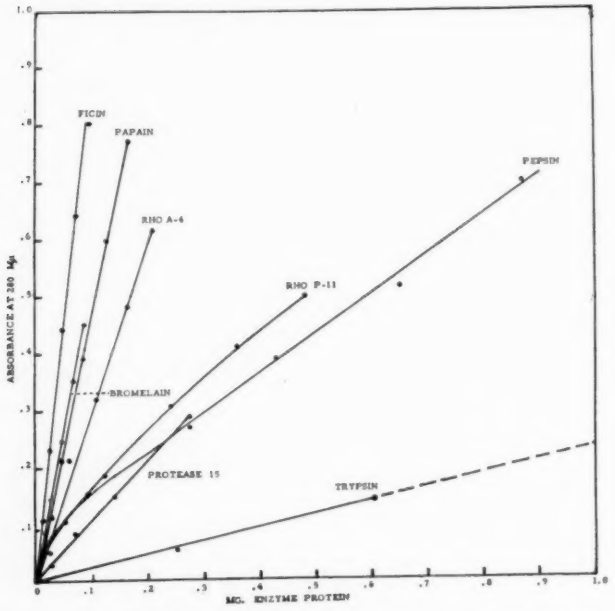

Figure 1. Comparison of proteolytic activity of enzyme preparations of different origin. The diluted enzymes were all activated with 0.1 M cysteine hydrochloride and held at 40° C for 1½ hours just prior to assay with hemoglobin substrate at pH 5.5.

A standard curve is constructed for each enzyme by plotting log of the viscosity in centipoise units versus the concentration of enzyme expressed in mg of protein.

Compatibility tests

Solutions of all possible paired combinations of plant, animal and microbiological proteases (Table 2) were made and held for 1½ hours at 25° C and at 40° C.

After 1½ hours the solution of each enzyme combination was assayed. The theoretical activity of the combination of enzymes was calculated by assaying the enzymes[b] individually under the same conditions of time, temperature, concentration and pH and then adding the absorbance for each enzyme in the case of the hemoglobin method (Table 1). In the gelatin assay procedure, the theoretical activity of each enzyme combination was estimated by adding the log reduction in viscosity of gelatin produced by each enzyme when assayed individually. As pointed out previously, the concentration of each enzyme in the enzyme combination was chosen so that the sum of the absorbances (hemoglobin assay) or the sum of the log reductions in viscosity of gelatin was in a measurable range as determined by the standard activity curve for each enzyme. The concentration range for each enzyme used in these studies is shown in Figure 1. The concentration scale was modified in order to give a composite as well as comparative picture of the range of concentrations used for each enzyme.

One hundred and sixteen enzyme assays were carried out in duplicate (232 determinations); 93.1% of the replicates deviate less than 5% from the mean absorbance

[b] The enzymes used in this study were obtained from the following sources:
Ficin—Paul-Lewis Laboratories, Inc.; Papain (Purified)—Paul-Lewis Laboratories, Inc.; Bromelain—Hawaiian Pineapple Co.; Rhozyme A-4—Rohm & Haas Co.; Rhozyme P-11—Rohm & Haas Co.; Protease 15—Rohm & Haas Co.; Trypsin—Wilson Laboratories; Pepsin—Cudahy Packing Co.

TABLE 1

Interaction of proteolytic enzymes in binary combinations[1] as percent inhibition

	Ficin	Bromelain	Rhozyme A-4 Fungal	Rhozyme P-11 Fungal	Protease 15 Bacterial	Trypsin	Pepsin
Papain	0.0[2]	− 6.6	− 4.5	−31.3	−18.3	−12.0	− 2.1
	−9.7[3]	− 1.5	− 9.6	−41.7	− 8.4	−20.3	−29.0
Ficin		−10.2	− 3.2	−38.5	− 9.2	−12.3	− 3.6
		−10.6	− 7.0	−39.6	−11.0	− 8.5	− 9.6
Bromelain			−10.5	−19.8	−11.8	− 6.0	−14.0
			−13.9	−23.5	−19.7	− 1.3	−35.7
Rhozyme A-4 Fungal				− 8.1	− 7.8	−21.1	−14.5
				−10.0	−13.8	−10.3	− 5.1
Rhozyme P-11 Fungal					− 8.5	−10.0	− 4.2
					−10.1	− 8.3	− 9.0
Protease 15 Bacterial						−14.4	−19.0
						− 1.2	− 6.4
Trypsin							− 3.6
							− 7.0

[1] Enzymatic interaction time limited to 1½ hours following which assays were conducted with hemoglobin substrate (see hemoglobin method).
[2] Upper figure in each square obtained for enzyme interaction at 25° C.
[3] Lower figure in each square obtained for enzyme interaction at 40° C.

TABLE 2

Summary of significant protease interactions[1]

25° C		40° C	
Protease combination	Per cent inhibition	Protease combination	Per cent inhibition
Ficin + Rhozyme P-11	38.5	Papain + Rhozyme P-11	41.7
Papain + Rhozyme P-11	31.3	Ficin + Rhozyme P-11	39.6
Trypsin + Rhozyme A-4	21.1	Bromelain + Pepsin	35.7
Bromelain + Rhozyme P-11	19.8	Papain + Pepsin	29.0
Pepsin + Protease 15	19.0	Bromelain + Rhozyme P-11	23.5
Papain + Protease 15	18.3	Papain + Trypsin	20.3
Pepsin + Rhozyme A-4	14.5	Bromelain + Protease 15	19.7
Trypsin + Rhozyme P-15	14.4	Bromelain + Rhozyme A-4	13.9
Bromelain + Pepsin	14.0	Rhozyme A-4 + Protease 15	13.8

[1] Interaction time limited to 1½ hours.

found for each assay. The maximum deviation from the mean was 7%. This deviation was noted in only one assay.

DISCUSSION

Synergistic action was anticipated in at least a few of the enzyme combinations evaluated. However, no synergism was manifested even at the highest temperature of interaction. In those experiments where interaction did occur, it was always in a negative direction. This phenomenon would indicate that the particular enzymes in the combination were either mutually digested by proteolytic action or were inhibited by substances, either enzymatic or non-enzymatic, in the enzyme preparations themselves. Regardless of the mechanism of interaction, the primary consideration was to demonstrate the lack of compatibility of certain proteolytic enzymes

that are produced commercially. The degree of interaction of all of the enzyme combinations evaluated is expressed as percent inhibition in Table 1.

In general, these data indicate that combinations of enzymes having the same origin (plant, animal, fungal or bacterial) did not show as significant an interaction as did combinations of enzymes of different origin.

The most significant enzyme interactions are summarized in Table 2 in order of decreasing interaction. Under the conditions of these experiments, it is obvious that the fungal proteases in combinations with either the plant proteases or with the animal proteases trypsin or pepsin manifested the most significant interaction.

It is interesting that there were no highly significant interactions between plant and animal proteases at 25° C. At 40° C, however, pepsin in combinations with either papain or bromelain showed a considerable degree of interaction. Upon consideration of the microbiological proteases as a group, the bacterial protease (Protease 15) did not exhibit as high a degree of interaction in combination with other proteases as did the fungal proteases.

Since the enzyme interactions shown in Table 1 were all evaluated by hemoglobin assay, reduction in viscosity of gelatin was used to further corroborate the interactions observed. With gelatin as a substrate for assay subsequent to enzyme interaction, papain and Rhozyme P-11 exhibited 24.7% inhibition at 25° C as compared with 31.3% inhibition found by hemoglobin assay. These results indicate that the interaction noted between these enzymes could not be attributed to inhibitors in the substrates used for assay.

In addition to measuring interaction of proteases at different temperatures, it was felt that the degree of interaction should be ascertained over a longer time interval than 1½ hours. Table 3 illustrates the extent of interaction at 25° C for a 24-hour period for each of the plant proteases, papain, ficin and bromelain in combination with the fungal enzyme Rhozyme P-11.

TABLE 3

Effect of interaction time on extent of protease interaction

Protease combination	Percent inhibition at 25° C	
	1½ hours	24 hours
Papain + Rhozyme P-11	31.3	39.0
Ficin + Rhozyme P-11	38.5	47.0
Bromelain + Rhozyme P-11	19.8	24.0

With each of these enzyme combinations a further increase in interaction was noted when the enzyme interaction time was increased from 1½ hours to 24 hours. These data indicate, however, that protease interaction occurred for the most part during the first 1½ hour period.

An insight into the mechanisms of protease interaction may be obtained upon examination of the data pertaining to the effects of time and temperature upon the extent of interaction. The protease interaction versus time studies indicated a definite increase in extent of interaction as the time of interaction was increased. These results would tend to support the hypothesis that mutual digestion through proteolytic action rather than inhibition was responsible for this interaction.

On the other hand, the protease interaction versus temperature studies indicated that both mechanisms could be operative. In 28% of the enzyme combinations evaluated less interaction was noted at 40° C than at 25° C. It is conceivable that a very heat labile inhibitor might be responsible in these cases. The majority of the enzyme combinations evaluated (72%) showed increased interaction at higher temperatures thus giving further credence to the mechanism of mutual digestion. Since most commercial proteases are known to contain enzymatic and non-enzymatic impurities, it is conceivable that one or both mechanisms could be responsible for the interactions observed.

On the basis of the foregoing data, it would appear that a very cautious evaluation of any protease combination should be made prior to commercial application using compatibility as an important criterion for selection.

SUMMARY

Binary combinations of proteases of plant, animal and microbiological origin show varying degrees of incompatibility as a result of enzyme interaction. This interaction was found to be most marked when certain proteases of different origin were combined. In most cases, interaction was found to increase with higher temperature indicating a mechanism of mutual digestion. In those cases where interaction was found to decrease with higher temperature, a heat labile inhibitor is suggested in lieu of or in addition to the mechanism of mutual digestion. Interaction was found to become more extensive as the time of enzyme interaction was increased from 1½ hours to 24 hours. This behavior would lend further credence to the mechanism of mutual digestion as an explanation for the observed interaction.

The significance of protease incompatibility is inferred in those commercial applications where combinations of proteases may be used such as in meat tenderizing formulas, chillproofing compounds for the brewing industry, etc.

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ORANGE PECTINESTERASE BINDING AND ACTIVITY[a]

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Pectinesterase (PE) of orange juice is associated with solid particles and is therefore a structure-bound insoluble enzyme *(4)*. The conditions for solubilization of PE from the solid particles, which include a requirement for the presence of salt and maintenance of the pH of the extracting solution at slightly alkaline values, have been well defined *(4)*. The nature of the binding of PE to the particles, which consist of cell walls, has not, however, been known. Furthermore, although PE is known to be active *in situ* under slightly alkaline conditions *(7)*, it has not been determined whether PE is active *in situ* under acid conditions of citrus fruit. This study concerns the nature of the binding of PE to cell walls and the activity of the enzyme so bound.

Aqueous suspensions of cell walls of high PE content were prepared from orange section membranes and juice sac walls. The progress curves of solubilization of the PE from such cell walls (at pH 7.5 and 0.15 *N* NaCl) and of deesterification of the pectin of the cell walls follow similar patterns. The temperature dependencies of the two rates are also similar. This suggests that deesterification of pectin precedes the solubilization of the PE and that the binding is of the nature of an enzyme-substrate complex. After complete deesterification of the cell walls an equilibrium is established between enzyme bound to cell walls and free enzyme in the extraction medium. A similar equilibrium is established on subsequent re-extractions. Thus even deesterified cell walls have an affinity for PE.

The addition of soluble pectin to the extracting medium and in the presence of salt causes solubilization of part of the PE of cell walls even at pH 3.8. At this pH, PE solubilization does not occur in the absence of added pectin. The solubilized PE acts on the solubilizing pectin and rapidly transforms it to a firm gel.

Cell walls firmly bind added orange PE in amounts up to 15 times greater than that naturally present. Crystalline α-chymotrypsin (α-Xt) is bound by cell walls in even greater amounts than is PE. The two enzymes compete for the same binding sites. Therefore, the binding is not specific for PE. Bound α-Xt, like bound PE, is dissociated from the cell walls by salt at slightly alkaline pH. Accordingly, such binding is apparently of an ionic nature. Removal of part or all of the pectic material from the cell walls by purified polygalacturonase results in corresponding loss in the binding capacity of the cell walls for both enzymes.

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[a] Work conducted under a cooperative agreement at the California Institute of Technology.

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The pH-activity relationship of the *in situ* action of the cell-wall-bound PE acting on cell wall pectin has been compared with that of soluble PE acting on soluble pectin. The two reactions possess the same pH optimum but the *in situ* enzyme is inactive at pH 4.5 and below, a pH range in which the soluble enzyme is still active. Accordingly, the cell-wall-bound PE is essentially inactive at the natural pH of citrus fruit.

MATERIALS AND METHODS

Enzymes and enzyme assays. The PE was a relatively crude preparation from orange flavedo *(4)* which was dialyzed against 0.05 *M* $NaHSO_3$ at 5° C for several days followed by water dialysis until the $NaHSO_3$ has been removed, and then lyophilized. The α-Xt[d] was recrystallized twice, dialyzed against water, and lyophilized. The highly purified polygalacturonase (PG) was that prepared from commercial pectinase using the procedure of acid treatment followed by ammonium sulfate and lead acetate fractionations *(3)*. PE was assayed by continuous potentiometric titration using slow-set pectin of 180 grade and containing 0.8% ash. Activities are expressed in pectinesterase units (PE units), i.e., milliequivalents of bonds hydrolyzed per minute per aliquot *(4)*. Xt was assayed by a similar procedure using tyrosine ethyl ester as substrate. Activities are expressed in the same type of unit (Xt units) *(1)*.

Nitrogen was determined by Nesslerization using ammonium sulfate as a standard. Pectic substances were determined as anhydrouronic acid (AUA) by the method of McComb and McCready *(6)*.

Juice sacs and segment membranes ("rag") were used for the preparation of orange cell walls. The "rag" from 10 lb of Valencia oranges was removed from the juice by filtration through cheese cloth and suspended in water to give a total volume of 3 liters. The suspension was comminuted batchwise in a blender for 5 min at room temperature. The resulting thick suspension was diluted with water to a total volume of 6 liters, further comminuted in an ice-water-cooled colloid mill at a close setting, and centrifuged at 15° C and 2000 rpm for 20 min. The supernatant was discarded. The cell wall preparation was suspended in water and recentrifuged 5 times. The washed cell walls were suspended in water to give a total volume of 1.5 liters and preserved with toluene. The dry weight of the preparation was 2.3% of the wet weight. AUA constituted 39% of the dry weight. The methyl ester content was 3.9% as determined by PE hydrolysis from which it may be calculated that 55% of the carboxyl groups of the AUA are present in the ester form. The preparation possessed a high PE content, 0.06 PE unit/ml suspension.

RESULTS

In situ PE action and PE solubilization. Both the presence of salts and a pH of approximately 7.5 are known to be required for solubilization of orange PE *(4)*. The time course of the solubilization under these conditions as well as the simultaneous liberation of pectic carboxyl groups is given in Figure 1 both for 0° and 30° C. It is evident that PE action *in situ* either precedes or occurs simultaneously with solubilization of the enzyme. The two reactions also possess essentially the same temperature coefficient. These results suggest that the binding of PE to cell walls is one of enzyme-substrate complex formation and that the enzyme is not solubilized until after hydrolysis of the cell wall substrate.

Distribution of PE between cell walls and extracting solution following *in situ* PE action. Even after deesterification of the cell wall pectic substances, the cell walls retain a strong affinity for PE (Table 1). At a concentration of 0.075 *N* sodium chloride and under the extraction conditions employed, the PE is approximately equally distributed between the extracting solution and deesterified cell walls. On subsequent extractions the distribution gradually increased in the extracting solution. If the salt concentration is raised to 0.15 *N* the distribution of PE between the solution and deesterified cell walls is increased to approximately 2.4 to 1, the distribution

[d] Purchased from the Worthington Biochemical Laboratory, Freehold, New Jersey.

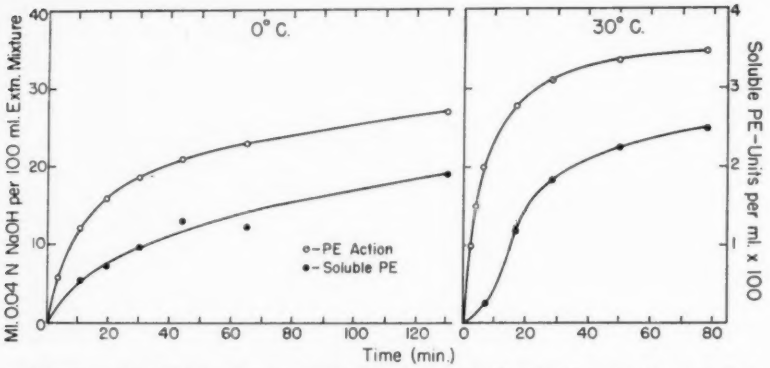

Figure 1. In situ PE action and PE solubilization at 0° and 30° C. The in situ action was measured by determining the amount of 0.04 N NaOH required to maintain pH 7.5 of the mixture of 100 ml of cell wall preparation plus 100 ml H_2O and a final concentration of 0.15 N NaCl. PE solubilization was measured by periodic removal of aliquots which were then centrifuged and the PE activity of the supernatant determined.

likewise increasing on subsequent extractions. Obviously deesterified cell walls possess a strong affinity for PE even under optimum extraction conditions.

Extraction of PE from cell walls with soluble pectin and salt. The initial cell wall suspension had been adjusted to pH 3.8 or essentially that of orange juice. At this pH, the addition of sodium chloride to a concentration of 0.15 *N* failed, even after an extended time (4 hr), to solubilize any detectable amount of PE. However, addition of soluble pectin, particularly in the presence of salt, did solubilize a part of the PE (Table 2). Less than 0.2% of the PE was solubilized in 20 min by approximately 1% pectin solution in the absence of added salt. Longer extraction periods failed to solubilize more enzyme. The addition of NaCl to the pectin extracting solution caused a marked increase in the amount of PE solubilized. In 0.15 *N* NaCl the amount of PE solubilized was 10 times as great as in the absence of salt. At NaCl concentrations (in the presence of pectin) of 0.3 and 0.6 *N* still more enzyme was solubilized. Two per cent pectin solution, 0.15 *N* with respect to NaCl, solubilized 2.2% of the PE as compared with 1.7% solubilized by 1% pectin solution. The pectin extraction solutions containing NaCl gelled in a relatively short time (Table 2), demonstrating that the solubilized PE had deesterified the soluble pectin.

TABLE 1

Effect of NaCl on distribution of PE between cell walls and extracting solution following in situ PE action

Extraction No.	0.075 *N* NaCl[1]		0.15 *N* NaCl[2]	
	Units PE solubilized	Ratio solubilized PE to remaining cell wall PE	Units PE solubilized	Ratio solubilized PE to remaining cell wall PE
1	12	1.0	4.32	2.4
2	6	1.1	1.36	3.0
3	3	1.2	0.36[3]	3.6
4[4]	1.6[5]	2.0		

[1] To 400 ml of cell wall preparation were added 400 ml of H_2O and the PE was extracted by adding NaCl to give a final concentration of 0.075 *N* and maintaining the pH at 7.5 by addition of 1 *N* NaOH. Subsequent extractions were made with the same volume of NaCl solution.

[2] To 100 ml. of cell wall preparation were added 100 ml of H_2O and the PE was extracted by adding NaCl to give a final concentration of 0.15 *N* NaCl and maintaining the pH at 7.5 by addition of 1 *N* NaOH. Subsequent extractions were made with the same volume of NaCl solution.

[3] Approximately 0.1 PE unit remained in the cell wall preparation after the third extraction.

[4] 1200 ml of 0.075 *N* NaCl were used for the fourth extraction.

[5] Approximately 0.8 PE unit remained in the cell wall preparation after the fourth extraction.

TABLE 2

Extraction of PE from cell walls with soluble pectin [1]

NaCl concentration	PE units extracted in 20 minutes	Amount of PE extracted
		%
0	0.0005	0.17
0.15 *N* [2]	0.0050	1.7
0.30 *N* [3]	0.0270	9.0
0.60 *N* [2]	0.0175	5.8

[1] The 5-ml samples of orange cell wall preparation were extracted at 25° with 45 ml of 1% soluble pectin containing the concentration of NaCl indicated. The suspensions were then clarified by centrifugation and aliquots of the supernatants assayed for PE.
[2] Extraction solution gelled in 18 hr.
[3] Extraction solution gelled in 30 min.

In situ and in vitro pH-activity relationship for PE. The above results suggest that PE is inactive *in situ* at a pH at which it is active *in vitro*. In order to study this further the pH-activity relationship of the cell-wall-bound PE acting on the pectic substance of the cell wall was determined and compared with the *in vitro* pH-activity relationship of PE acting on soluble pectin at optimum concentration. The results

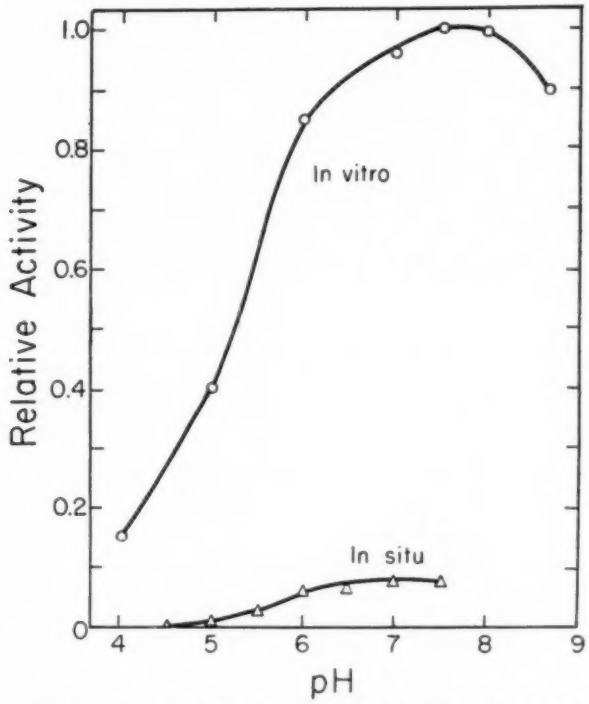

Figure 2. In situ vs. in vitro pH-activity relationship. The in situ pH-activity relationship was measured by determining the amount of 0.02 N NaOH necessary to maintain the desired pH of a mixture of 10 ml of cell walls and 10 ml of 0.30 N NaCl solution, the inherent pectic substances of the cell wall serving as the sole substrate. The in vitro curve was previously obtained at optimum soluble pectin concentration (0.5%,4). At acid pH values the proper correction for incomplete titration was applied.

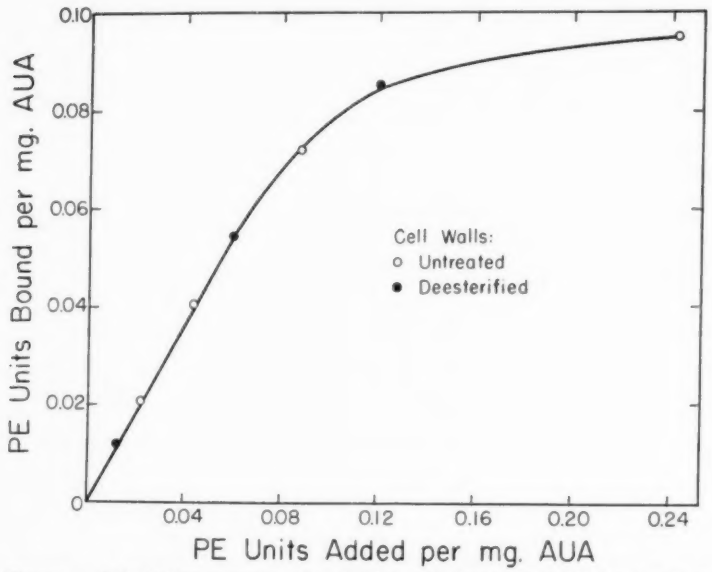

Figure 3. PE binding capacity of untreated and deesterified (PE-free) cell walls. Cell wall suspensions, either untreated or deesterified, were diluted with an equal volume of water and cooled to 0° C. Weighed samples of the partially purified PE were dissolved in the suspension. After 2 hr the suspensions were centrifuged and aliquots of the supernatants were assayed for PE. The deesterified cell walls were those from which the PE had been extracted with 4 successive extractions with 0.075 N NaCl solution (Table 1) and the pH of the suspension was adjusted to 4.0. The cell walls were centrifuged and resuspended in water.

are given in Figure 2. In this figure use is made of relative activity. A relative activity of unity corresponds to the activity which would be observed if all of the cell wall enzyme were solubilized and then allowed to act on 0.5% pectin solution *(4)*. The pH optima for *in situ* and *in vitro* activity are identical (pH 7.5). The difference in relative activities for the two kinds of action is undoubtedly due to limiting substrate concentration and insolubility of the PE in the case of the *in situ* action. Most noteworthy is the fact that no *in situ* PE activity occurred at pH 4 to 4.5 at which PE *in vitro* still possesses relatively great activity. At the natural pH of orange juice, therefore, the cell-wall-bound PE would appear to be essentially without action although the enzyme in solution at this pH would act on soluble pectin.

PE-binding capacity of cell walls. The PE-binding capacities of cell walls and of deesterified (PE-free) cell walls were determined using the partially purified PE preparation (Figure 3). As measured by assays of the supernatants essentially all of the PE added, up to a concentration of 0.07 PE unit per mg cell walls AUA, was absorbed by the cell walls in 2 hr. Incubation times of only 4 min resulted in adsorptions which were only slightly lower than those after 2 hr. Untreated and deesterified cell walls behaved identically. The maximum amount of PE adsorbable was approximately 0.10 unit per mg AUA, an amount 15 times greater than the native PE content of cell walls. The adsorbed PE was firmly bound since repeated water washings failed to solubilize any of the enzyme. At the natural pH of the cell wall suspensions (pH 4.0), addition of sufficient NaCl to make the suspension 0.15 *N* caused the solubilization of a small amount of PE. For example, such treatment of deesterified or of untreated cell walls which had adsorbed 0.054 and 0.072 PE units per mg AUA, respectively, resulted in the solubilization of 5% of the enzyme, as deter-

TABLE 3

Purification of PE by cell wall adsorption

Enzyme preparation	Specific activity (PE units/mg N)
Partially purified PE	0.041
PE removed after cell wall adsorption	0.32
PE extracted from cell walls	0.56
Highly purified PE *(5)*	1.70

mined by PE assay of the supernatant after 15 min. On the other hand, at pH 7.5 and at the same NaCl concentration over 90% of the adsorbed enzyme was solubilized in 15 min. It is obvious that orange cell walls are capable of firmly binding added PE in amounts much greater than those normally present.

The curve which relates PE binding to the concentration of added PE (Figure 3) is reminiscent of that for binding of substrate by enzyme. PE binding may therefore be treated formally by Michaelis-Menten kinetics and Km value (dissociation constant of the enzyme-substrate complex) computed. The cell walls become saturated with the enzyme at sufficiently high PE concentration. At such saturation, an amount of the cell walls containing 1 mg AUA binds about 0.1 PE unit. The comparable value for the binding of PE by soluble pectin is 6.2×10^{-4} PE unit per mg AUA. Thus cell wall pectin *in situ* possesses the ability to bind 160 times as much PE as an equal amount of soluble pectin.

Purification of PE by cell wall adsorption. The partially purified, lyophilized PE used for adsorption studies possessed a specific activity of 0.041 PE unit per mg N (Table 3). The specific activity of highly purified orange PE is 1.70 *(5)*. The question might be asked whether adsorption of PE by cell walls is sufficiently specific as to affect an enrichment of PE from an impure mixture. Table 3 shows that adsorption of PE by cell walls and elution therefrom resulted in a purification of the enzyme and that, in fact, the specific activity of the PE was increased 8-fold. However, the adsorption was by no means completely specific since the specific activity of the eluted enzyme (0.32 PE unit per mg N) is still only one-fifth that of highly purified PE (1.70 PE units per mg N). Enzyme solubilized directly from

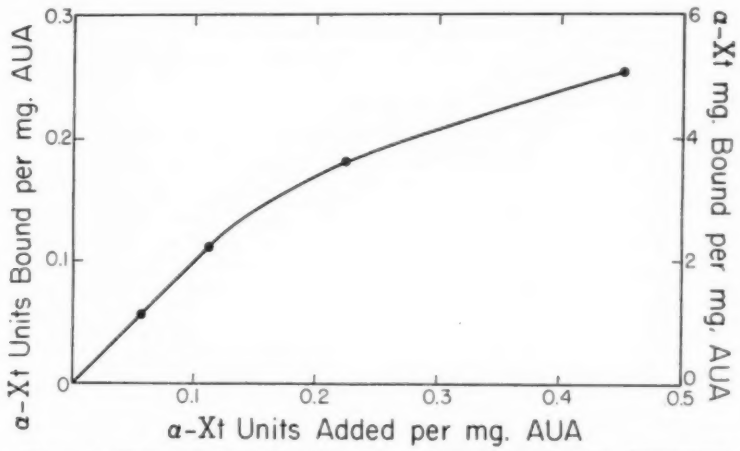

Figure 4. Chymotrypsin binding capacity of deesterified cell walls. Suspensions of deesterified cell walls were diluted with an equal volume of water and cooled to 0° C. Weighed samples of lyophilized α-Xt were dissolved in the suspension. After 2 hr the suspensions were centrifuged and aliquots of the supernatants were assayed for Xt.

the original cell walls by extraction with 0.075 *N* NaCl at pH 7.5 (Table 1) was dialyzed against cold water and lyophilized. The specific activity of this preparation was 0.56 PE unit per mg N, showing that other proteins accompany the cell walls. However, the fact that the specific activity of PE eluted from cell walls is approximately one-third that of the highly purified PE suggests that the enzyme thus obtained would provide an excellent starting material for preparation of pure PE.

Chymotrypsin (α-Xt) binding capacity of cell walls. Further evidence that the binding of enzymes by cell walls is not specific was obtained through a study of the binding of α-Xt to deesterified cell walls. The results (Figure 4) demonstrate that on the basis of enzyme activity the cell walls bind more Xt than PE (Figure 3). The specific activities of Xt and of highly purified PE are approximately the same *(2)*. Cell walls therefore bind more pure α-Xt than crude PE. Since the Xt was a pure crystalline preparation, the mg of α-Xt bound per mg of AUA can be and is indicated in Figure 4. At the highest concentration studied, 5 mg of enzyme protein were bound per mg of AUA, a strikingly large amount. The α-Xt was as firmly bound as was the PE since repeated water washings failed to remove it. α-Xt could however be completely eluted under the conditions identical with those required for elution of PE. For example, by treating the cell wall preparation which had adsorbed 0.06 Xt unit per mg AUA with sufficient NaCl to make the suspension 0.15 *N* and then adjusting the pH to 7.5, 90% of the enzyme was solubilized in 10 min as measured by assay of the supernatant after centrifugation.

Adsorption of α-Xt to cell walls blocks subsequent adsorption of PE. This was demonstrated by treating deesterified cell walls with sufficient α-Xt to cause the adsorption of 0.14 α-Xt unit per mg AUA (approximately one-half saturation). The cell walls were then centrifuged down, washed 3 times with water, and suspended in water to the original volume. PE was added to the suspension in the ratio of 0.035 unit per mg AUA (an amount which would be completely adsorbed in the absence of α-Xt, Figure 3) and then incubated at 0° C for 15 min. Assays of aliquots of the supernatant after centrifugation revealed that only 20% of the PE had been adsorbed. The two enzymes are, apparently, adsorbed on the same sites and interfere with one another in their adsorption.

Pectin material of the cell walls responsible for enzyme binding. In order to determine whether or not the AUA of cell walls is responsible for the binding of enzymes deesterified cell walls were hydrolyzed with highly purified PG so as to remove the AUA. For this purpose, deesterified cell walls were suspended in 0.2 *N* acetate buffer at pH 5.0, purified PG added, and the mixture incubated at room temperature with toluene as a preservative. Aliquots were removed periodically and the extent of reaction measured by determining the AUA in the supernatants. The action of PG on the *in situ* AUA was extremely slow as compared to its action on soluble pectinic acids as illustrated by the following experiment:

To 50 ml of deesterified cell walls were added 10 ml of water, 20 ml 1 *N* acetate buffer at pH 5.0, 20 ml of 0.5% disodium ethylene diamine tetraacetic acid, and 10 mg of purified PG (0.2 PG unit). This amount of PG was sufficient to completely hydrolyze an amount of soluble pectinic acid equal to the cell wall pectic substance in 1 hr. In the case of the cell walls, analysis of the supernatant after 1 hr revealed that only 12% of the AUA had been solubilized. After 1, 2, and 7 days, 24, 38, and 48%, respectively, were solubilized. Another 20 mg of purified PG were then added to the mixture and after an additional 5 and 8 days, 59 and 62%, respectively, had been solubilized. Addition of another 30 mg of enzyme and 14 additional days of incubation resulted in solubilization of 85% of the total AUA. Still another addition of 30 mg of PG and 5 more days of incubation resulted in 95% solubilization of AUA. The PG-treated cell walls were then centrifuged and washed several times by suspension in water and centrifugation. The washed, treated cell walls were next suspended in 50 ml of water. AUA analyses of aliquots of this suspension confirmed that 95% of the AUA had been removed. Another preparation of similar material was made in which 70% of the AUA had been removed. These preparations were compared with the original deesterified cell walls as to their enzyme-binding capacity, using concentrations of PE and α-Xt which would give essentially saturation of the original cell walls. The results (Table 4) show a relationship between the AUA content of the cell walls and their binding capacity for both enzymes. The

enzyme-binding sites of cell walls appear therefore to involve AUA. However, since highly purified PG is not electrophoretically homogeneous *(3)*, other polysaccharide-binding sites may also have been removed by the action of the PG preparation.

DISCUSSION

Over the past several years considerable effort has been devoted to the stabilization of frozen orange juice concentrates by heating to partially inactivate the PE. In these studies the amount of enzyme destroyed has been the only factor studied, and no attention has been given to effects on the substrate. However, heat treatment could result not only in PE destruction but also in changes in pectic material, such as partial solubilization of the latter from the suspended matter (cell walls). It is clear from the present results that cell walls firmly bind PE; that, in the bound state, the enzyme is inactive at the pH of orange juice on the *in situ* pectic material; that soluble pectin in combination with salts extracts part of the

TABLE 4

Effect of removal of AUA on enzyme binding by orange cell walls

Residual AUA	Binding capacity	
	PE	Xt
%	%	%
100	100	100
30	38	
5	6	12

PE from cell walls; and that the PE so solubilized hydrolyzes soluble pectin to an extent that causes gelation. The results suggest that gelation and possibly cloud loss of frozen orange juice concentrates might be caused by solubilization of pectin into the serum during processing. Proof of this would require a systematic study of the variations in soluble pectin as related to concentrate stability.

SUMMARY

During the solubilization of PE from orange juice cell walls (finely suspended "rag") at pH 7.5 in the presence of 0.15 *N* NaCl, deesterification of the cell wall pectic material and solubilization of the enzyme proceed at rates parallel to one another. Temperature changes also affect the two rates similarly.

After complete deesterification of cell wall pectin an equilibrium is established between enzyme bound to cell walls and free enzyme. An equilibrium is obtained on subsequent re-extractions. Thus even deesterified cell walls have a strong affinity for PE.

The solubilization of cell-wall-bound PE is promoted with addition of soluble pectin, provided salt is also present. The PE so solubilized rapidly hydrolyzes the soluble pectin with resultant gel formation.

The pH-activity relationship of the bound PE acting on cell-wall pectin has been compared with that for soluble enzyme acting on soluble pectin. Both have pH optima at 7.5. The bound enzyme is, however, inactive

at pH 4.5 and below. The soluble enzyme is still active in this pH region.

Cell walls, either untreated or deesterified, in suspension at pH 4 firmly bind added PE in amounts up to 15 times that naturally present.

An 8-fold purification of partially purified PE could be effected by adsorption on cell walls followed by elution. The purity of the enzyme thus prepared is approximately one-fifth that of the purest enzyme previously known.

Binding of PE to cell walls is not specific, since even greater amounts of chymotrypsin are bound just as firmly. Approximately 5 mg of this enzyme can be bound by each mg of pectic material in cell walls.

A loss in ability of the cell walls to bind either enzyme occurred when pectic material was removed by treatment with highly purified polygalacturonase.

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BIOCHEMISTRY OF CHICKEN MUSCLE AS RELATED TO RIGOR MORTIS AND TENDERIZATION[a]

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The post-mortem tenderization of meat is a problem of great importance to the meat and poultry industries as well as a matter of considerable theoretical interest to the biochemist and biophysicist. The present paper concerns the biochemical changes occurring in chicken muscle post-mortem and their relation to the development of tenderness. Other papers from this laboratory have dealt with variables in poultry processing and their effect on tenderness *(13, 24)*.

The chemical and physical reactions that occur in muscle post-mortem have been investigated by Bate-Smith and Bendall *(1, 3, 4, 5)* and by several groups studying muscle contraction. Experiments performed on rabbit muscle by Erdös *(8)* and confirmed by Bate-Smith showed that the chemical event most closely linked with the onset of rigor mortis is the disappearance of adenosine triphosphate (ATP). In living, respiring muscle, a high level of ATP is maintained by oxidation of organic compounds. When the animal dies and oxygen is no longer available to the cells, ATP (and creatine phosphate, a reservoir of phosphate-bond energy) can be maintained only by anaerobic glycolysis. After the muscle glycogen has been utilized, the ATP concentration decreases rapidly and the muscle passes into rigor mortis, a state characterized by a sharp increase in the modulus of elasticity of the muscle and the development of a rigid, non-plastic texture. Bendall *(5)* demonstrated the disappearance of creatine phosphate before there were any changes in muscle texture and concluded that this compound played no part in the development of rigor mortis other than as a reserve of phosphate-bond energy. The production of lactic acid from glycogen causes the pH of meat (initially at pH 7 or above) to drop to final levels of 5.5 to 6.0. The chemical and physical changes described above are more fully reviewed by Bate-Smith *(2)*.

As a first step in elucidation of the biochemistry of rigor mortis and its relation to tenderization in fowl, metabolites known to be subject to rapid post-mortem change in other species (glycogen, lactic acid [pH], ATP, and creatine phosphate) have been followed in freshly slaughtered chicken muscle under various environmental conditions. In addition, changes in the modulus of elasticity as the muscle passes into rigor mortis have been followed under these same conditions. Finally, the effects of these environmental conditions on the final tenderness of muscle have been determined. The studies were conducted on meat-type chicken fryers, generally 3 to 4 pounds in weight.

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[a] A preliminary report on this work was presented at the Poultry Science Association meeting, Ithaca, New York, August, 1958.

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EXPERIMENTAL

Chemical analyses. All chemical determinations, as well as extensibility measurements and evaluations of toughness, were made on the *Pectoralis major*, the large breast muscle. Glycogen was determined by the method of Seifter *et al. (26)* with the reagent stability improved by addition of 1% thiourea *(25)*. Tissue extracts for the determination of ATP were prepared by homogenizing, in a blender, 2 to 3 g of muscle in 10 times its weight of 5% trichloroacetic acid (TCA) and filtering. ATP was determined by the method of Griswold *et al. (11)*, except that the labile phosphate from ATP was separated by precipitation as $MgNH_4PO_4$ after its hydrolysis in 1.0 *N* H_2SO_4 for 15 minutes at 100° C *(19)*. The pH of muscle was determined with a Beckman Glass Electrode pH Meter, Model GS, on a 10 to 12 g sample of muscle, homogenized in a blender, in twice its weight of 0.005 *M* sodium monobromoacetate *(3)*.

Extensibility measurements. Measurements of the relative extensibility of muscle strips were made with equipment similar in principle to that of Bate-Smith *(1)* and Bate-Smith and Bendall *(4)*. The displacements were sensed and recorded by electrical rather than mechanical means. A muscle strip went through 4 complete cycles of loading and unloading during each hour, the load being applied or removed for 7.5-min periods by a timer-operated mechanism. The muscle strips were very close to 1 cm^2 in cross section and from 4 to 6 cm in length. The modulus of elasticity is expressed in terms of grams per cm^2.

Evaluation of toughness. The relative toughness of the meat was determined by shear force measurements on the whole muscle or a portion thereof. A Warner-Bratzler shear force apparatus was used *(6)*. These measurements have been shown by a number of investigators to be correlated with organoleptic ratings [cf. Klose *et al. (13)*].

The meat was cooked before shearing, either by frying an entire half-chicken in hot vegetable oil or by removing the raw muscle from the carcass and cooking in boiling water. When the time the birds were allowed to remain above the freezing point was critical, the birds were cooked from the frozen state. When excised muscles were cooked, the frozen carcasses were allowed to thaw to the minimum extent necessary for removal of the *Pectoralis major* muscle, which was then cooked in boiling water. To prevent uncontrolled distortion of the separated muscle while boiling, the muscles were clamped in a special mold. The mold consisted of two plates of 0.091-gauge duralumin, 7½ in wide by 14 in long, clamped together by machine screws with wing nuts. The plates were maintained a fixed distance apart by tubular spacers around the screws. After boiling 30 min, the cooked muscles were cooled for a short time in running tap water and then held in a refrigerator until sheared. Strips one inch wide and parallel to the fibers were cut from each muscle. This provided a strip of uniform 2.5 cm^2 cross-section for shear force measurement. Three to 6 individual determinations could be obtained from each strip.

RESULTS

Comparisons with other species. The time of onset of rigor mortis, the initial ATP content, and the ultimate pH of chicken muscle are compared in Table 1 with data in

TABLE 1

Comparative data on rigor mortis

Species [1]	Time of onset of rigor mortis (hr)	Ultimate pH [2]	Initial ATP content (mg/g fresh tissue)	Literature reference
Chicken	2–4.5	5.8–5.9	4.8	
Rabbit	1.5–4	5.9	4.1	*3, 4, 5*
Rat	....	5.9	....	*4*
Pig	....	5.4	....	*2*
Whale	14–50	5.5–5.9	3.0	*21*
Beef	10	5.4	3.1	*22*
Beef—37° C	4	5.4	3.1	*22*
Horse—37° C	3.5–4.5	5.5–6.0	2.3–3.2	*17, 18*

[1] Temperatures of samples were falling during onset except as noted.
[2] The steady pH attained when rigor mortis is fully established.

the literature for other species. Values are similar for the several species but large animals tend to require more time for the onset of rigor mortis. The relatively high level of ATP in chicken muscle is in accord with data of Krishnan and Nelson *(16)*, who were able to isolate 50 to 80% more ATP from turkey white muscle than from rabbit or lamb.

Analyses for creatine phosphate in chicken have shown, in confirmation of work done by Bendall on rabbit muscle *(5)*, an extremely rapid breakdown before any change in muscle texture occurs.

Changes in extensibility observed for chicken muscle were similar to those reported by other workers for beef or rabbit. The modulus of elasticity was generally about 1 to 2 x 10^5 g/cm^2 in the pre-rigor condition. This value increased 5- to 10-fold when rigor was fully established. Concurrent estimations of glycogen and ATP demonstrated the same sequence of chemical and physical events in chicken muscle that had already been established for other species. Figure 1 shows the time course of the ATP concen-

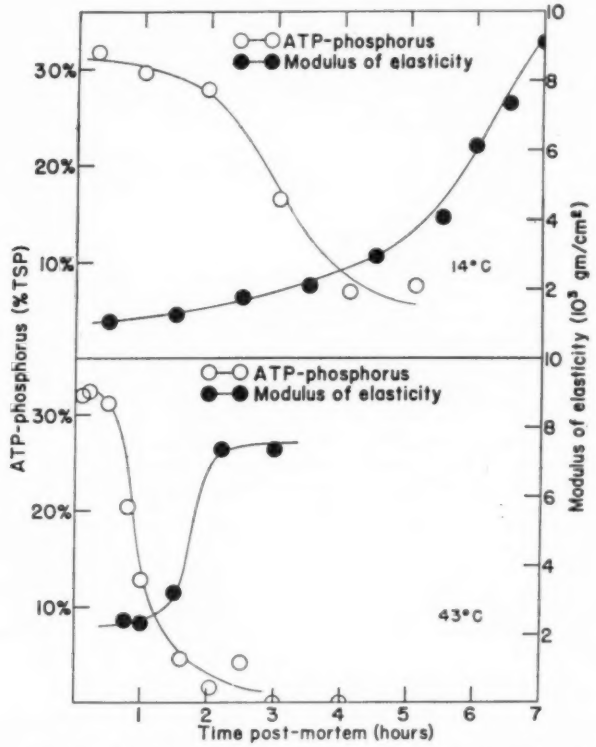

Figure 1. Relation between post-mortem changes in ATP concentration and modulus of elasticity at two temperatures.

tration and the modulus for typical muscle strips at 14° and 43° C. At the lower temperature the strip remained plastic for a considerable period of time during which the ATP concentration was decreasing rather rapidly.

In general, when about two-thirds of the ATP has disappeared, the muscle strip begins to lose its plasticity. The loss of plasticity or extensibility continues for a con-

siderable time, as much as 8 hr in some cases. Muscle strips placed in the apparatus to thaw after having been frozen in the pre-rigor condition, or strips subjected in the apparatus to temperatures at which proteins are rapidly denatured, shorten markedly, often to the extent of 50%. This shortening is accompanied by a simultaneous loss of extensibility.

As with rabbit *(1)* and beef *(22)*, the loss of extensibility that occurred during the onset of rigor was not reversed during the resolution of rigor. In one experiment, fryer chicken muscle reached a minimum extensibility at 8 hr post-mortem, then remained unchanged for an additional 22 hr. This is well beyond the time when rigor is resolved in chicken and is also beyond the time for development of full tenderness as far as aging is concerned. As pointed out by Marsh *(22)*, whatever the resolution of rigor is, it does not involve changes in the elastic properties of muscle.

Effect of cutting the muscle pre-rigor. In most of the experiments described in the following sections, the *Pectoralis major* muscles of freshly slaughtered chickens were excised as rapidly as possible. Each of two different experimental treatments was then accorded to the two muscles from the same bird. Any difference in tenderness could then be ascribed to the treatment, as paired muscles from the same bird are remarkably alike in their tenderness if they have undergone identical treatments.

From the work of Lowe *(20)* and Koonz *et al. (14)*, it was anticipated that cutting or excising muscles before onset of rigor mortis would induce a toughness which would be only partially resolved by aging. In order to measure the extent of this effect, 8 cockerels were killed by bleeding, scalded at 53° C (127° F) for 60 sec, picked on a single-drum feather picker, and eviscerated immediately. One *Pectoralis major* muscle from each bird was removed, sealed in a polyethylene bag, and replaced under the skin. The skin was then fastened in place by means of a strip of masking tape and the birds were placed in freely draining cracked ice. Twenty-four hr post-mortem, the remaining *Pectoralis major* muscles were removed and all muscles were cooked in boiling water before determination of their shear values.

As expected, the muscles that had been excised prior to onset of rigor mortis were less tender than their controls. The mean shear value of the cut muscles was 12.6 lb compared to 5.8 lb for the controls. Statistically, this difference is very highly significant ($P < 0.001$). However, in the following sections, it will be seen that additional treatments superimposed upon the cutting of pre-rigor muscle can induce mean shear values in excess of 30 lb. Thus, it is felt that muscle excised before onset of rigor mortis is suitable for studies of additional treatments even though excising induces a moderate degree of toughness.

Effect of mechanical stimulation. Pool *et al (24)* have reported on the toughening effect of mechanical feather pickers. The action of these machines, which have stout rubber fingers on a rapidly revolving drum, is to make the subsequently cooked meat markedly less tender than hand-picked controls. This effect had previously been noted by Klose *et al. (12)* and Wise and Stadelman *(30)*. In order to investigate chemical changes accompanying this effect, a group of eight 11-week-old cockerels was slaughtered under commercial conditions (starved overnight, stunned by electric shock, bled with an outside neck cut for 2 minutes, scalded at 53° C (127° F) for 50 sec, feathers removed with a single-drum picker, eviscerated immediately, and placed in an ice-water bath) and compared to a control group subjected to identical conditions with the exception that feathers were removed by hand. At 2.5 hr post-mortem, the average ATP-phosphorus content of muscle from the beaten birds was 5.9% of the total TCA-soluble phosphorus (TSP) while that of the hand-picked group was 23.8%. The probability that this result was due to sampling error was less than 0.001. When this experiment was repeated using a sub-scalding temperature of 60° C (140° F) for 50 sec the results were similar. Muscles from the beaten group had an average ATP-phosphorus content of 5.2% of the TSP, while the hand-picked controls had a value of 14.7% of the TSP. For this experiment also, $P < 0.001$.

Study of this effect was extended to a paired-muscle experiment. A chicken was killed by bleeding and both *Pectoralis major* muscles were removed immediately. One muscle was struck gently but repeatedly with a wooden mallet for 2 min. Then both muscles were placed in polyethylene bags and held in running tap water (15° to 16° C). Samples were removed periodically for ATP and pH determinations. (Control experiments showed that there was no difference in the rates of ATP disappearance or pH change between

the left side and the right side when both were treated identically). Figure 2 shows that both the pH and ATP dropped more rapidly in the muscle that had been beaten. The time required for the ATP concentration to fall to 50% of its initial value was reduced from 3.2 to 2.3 hr.

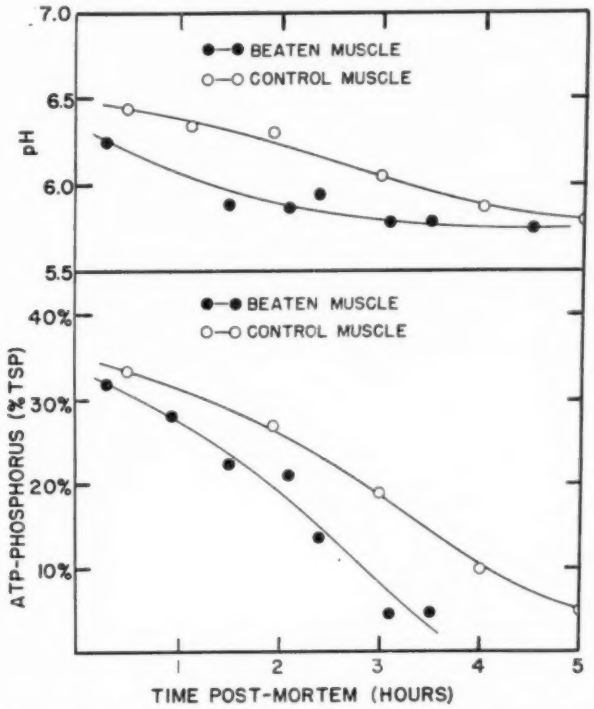

Figure 2. Effect of mechanical beating on rate of change of pH and ATP in paired muscles.

The effect of beating on subsequent toughness was demonstrated for excised muscle as follows: Two chickens were killed by bleeding and both *Pectoralis major* muscles were removed from each bird. One muscle from each bird was struck gently but repeatedly for 2 min. All 4 muscles were then stored in polyethylene bags for 24 hr at 2° C prior to cooking. The average shear value of the beaten muscles was 12.6 lb compared to 4.7 lb for the control muscles. It is evident from these experiments that the severe mechanical handling of fresh muscle tissue that induces muscle toughness *(24)* leads to rapid rates of ATP disappearance and pH change.

Effect of post-mortem temperature. The marked effect of temperature on post-mortem changes, as illustrated in Fig. 1, was systematically investigated in the range from 0° C to 40° C in 10 degree increments. For determining the rates of ATP disappearance, the *Pectoralis major* muscles were removed from freshly killed birds and placed separately in polyethylene bags.

Each member of a pair was held at one of 2 temperatures which differed by 10 degrees, and sampled periodically for ATP. Four temperature comparisons were made, viz. 0° vs. 10°, 10° vs. 20°, 20° vs. 30°, and 30° vs. 40° C. The results of a typical series are shown in Figure 3. The unexpected observation that the ATP disappears more

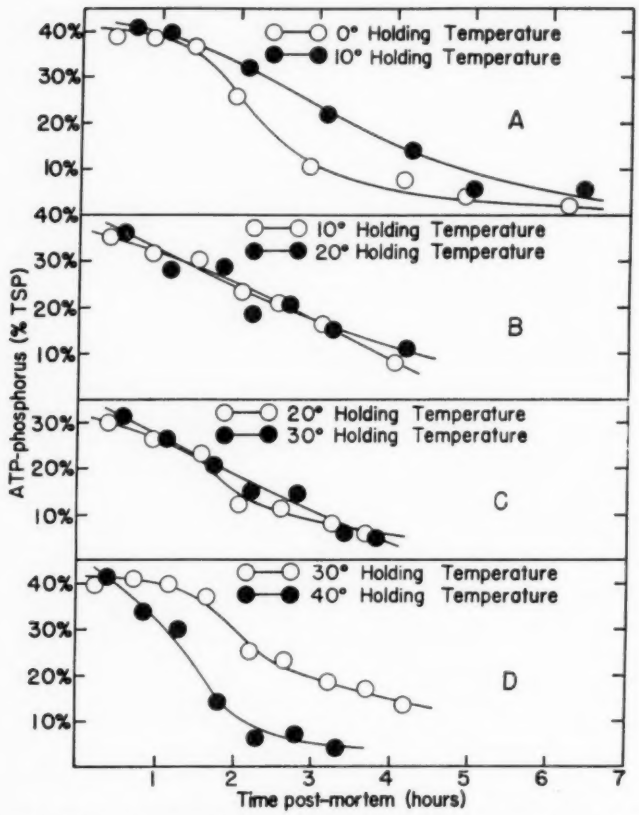

Figure 3. Effect of post-mortem holding temperature on rate of change of ATP in paired muscles. A—0° vs. 10°. B—10° vs. 20°. C—20° vs. 30°. D—30° vs. 40°.

rapidly at 0° than at 10° has been confirmed by replicate experiments. Additional tests at 43° C (109° F) and at 52° C (126° F), which fall in the range of commercial scalding temperatures, showed a rapidly increasing rate of ATP disappearance. Time required for 50% reduction in initial ATP concentration at 40°, 43°, and 52° C was 1½, 1, and ¼ hr, respectively.

The effect of post-mortem temperature on muscle toughness was investigated with 12 cockerels for each 10-degree increment from 0° C to 40° C. The *Pectoralis major* muscles from each bird were placed in individual polyethylene bags. Each member of a pair was held at one of two temperatures which differed by 10 degrees. Four temperature comparisons were made: 0° vs. 10°, 10° vs. 20°, 20° vs. 30°, and 30° vs. 40° C. After 4 hours' incubation at these temperatures, all muscles were stored at 2° C for an additional 20 hr before cooking and shearing.

Results of these shear force measurements are presented in Table 2. To examine the relationship of toughness to rates of ATP disappearance (Fig. 3), values for each were transformed by expressing them as ratios and setting the value for 0° at 100%. These values are plotted in Figure 4. (The "rate of ATP disappearance" is arbitrarily defined as the reciprocal of the time required for the ATP concentration to fall to 50% of its initial value.) The fact that both curves for these excised muscles follow the same

TABLE 2

Effect of post-mortem temperature on muscle toughness

Temperature comparison	Mean shear value		Mean pair difference [1]	P [2]
	Higher temperature	Lower temperature		
°C	lb	lb	lb	
0° vs 10°	7.5	11.0	−3.5	<0.001
10° vs 20°	6.7	4.7	+2.0	<0.001
20° vs 30°	9.4	6.8	+2.5	<0.01
30° vs 40°	19.6	11.3	+8.3	<0.001

[1] Each value represents the mean difference of the 12 muscle pairs, the value for the lower temperature being subtracted from that for the higher.
[2] Probability that the mean pair difference is due to sampling error.

general course and show the unexpected minimum in the 10° to 20° region is strongly indicative of a correlation between rate of ATP disappearance and muscle toughness.

The effect of post-mortem temperature on toughness of birds held in the whole, eviscerated state was also investigated. Several groups of cockerels were slaughtered under commercial conditions (see "Results, Effects of Mechanical Stimulation") and

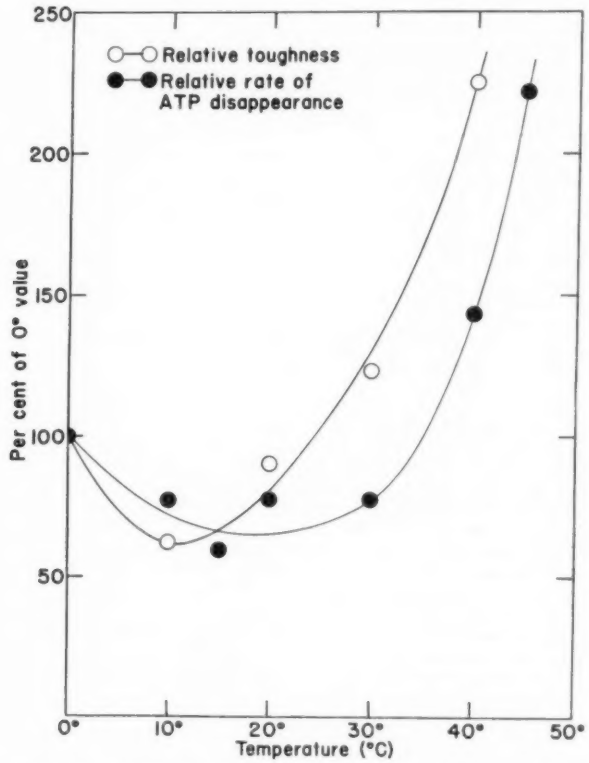

Figure 4. Relative toughness and relative rate of ATP disappearance in muscles held at various temperatures post-mortem.

aged for 4 hr in water baths at various temperatures from 0° to 40° C. Following this, the birds were packaged in individual polyethylene bags, frozen immediately, and cooked from the frozen state in deep fat before determination of their shear values. There was no significant difference between any of the groups. When the experiment was repeated by aging 4 hr at the experimental temperature and holding an additional 20 hr at 0° C before packaging and freezing, again there was no significant difference in shear value between any of the groups. It is not known why these results are different from those on excised muscle, but it may be assumed that the variation between birds, which is larger than that between paired muscles from the same bird, is in part responsible for the lack of significance.

In the studies of beating and temperature effects just described, a rapid rate of breakdown of ATP was accompanied by a decreased tenderness of the aged poultry meat. These observations lead to the speculation that this correlation is generally true. Such a circumstance implies that accelerated depletion of ATP interferes with the normal course of rigor mortis not only by hastening its onset but also by altering its resolution in such a manner that normal tenderness is not achieved. The general validity of the foregoing correlation seems established by studies of 4 additional factors that influence ATP disappearance.

Other factors that affect ATP disappearance

Thaw rigor. The rapid development of rigor mortis that occurs when a living muscle is frozen and thawed has been known for many years, but not until 1950 was there any systematic attack made on its fundamental biochemistry. In that year, Perry *(23)* and Godeaux *(10)* independently published results of experiments on frog and rabbit muscles. Godeaux *(10)* and Crepax and Hérion *(7)* showed that all of the ATP present in the frozen tissue is broken down within 30 min of thawing. More recently, Szentkirályi *(28)* showed that the half-time of ATP dephosphorylation in thin strips of thawed rabbit muscle is approximately one minute. This extremely rapid dephosphorylation is accompanied by a deamination with the production of inosinic acid.

The chemical changes occurring during thaw rigor in chicken muscle were followed by the techniques already described. The *Pectoralis major* muscles were removed rapidly from a freshly killed chicken and placed in polyethylene bags. One muscle was frozen immediately in a dry ice-ethanol bath and stored at −23° C (−10° F); the other muscle was placed in tap water (13° to 14° C) and sampled periodically for ATP and glycogen. The frozen muscle was sampled within 2 days for ATP and glycogen while frozen, thawed in tap water, and sampled periodically. The results are shown in Figure 5. "Time post-mortem" refers, in the case of frozen-thawed muscle, to total post-mortem time that the muscle is held at temperatures above freezing. The lower glycogen content in the frozen sample may be due to loss of glycogen during analysis of the tissue (2 g frozen muscle dropped into 4 ml of 30% KOH in a boiling water bath). In any case, the disappearance of both glycogen and ATP is much more rapid in the thawed muscle than in the unfrozen muscle. The rapid breakdown of glycogen that follows freezing and thawing was confirmed by following the rate of pH decrease in muscle samples treated similarly to those just described except that the holding temperature was 0° C (Figure 6). It is not known why the ultimate pH of this chicken was so high (6.2 to 6.3). The increased rate of pH change in chicken muscle following freezing and thawing differs from the results of Fuhrman *(9)*, who observed that the rate of anaerobic glycolysis in several tissues of the rat, including muscle, is reduced by freezing and thawing.

An experiment was performed to determine the effect of freezing excised muscle on toughness of the subsequently cooked meat. Twenty-three cockerels were divided into 2 groups of 11 and 12 birds each. They were killed by bleeding, and the *Pectoralis major* muscles were removed immediately and sealed in individual polyethylene bags. One muscle from each bird in the first group was frozen rapidly in a dry ice-ethanol bath, thawed immediately in running tap water, and aged with the remaining muscles from both groups for 24 hr at 2° C. Just before cooking, one muscle from each bird in the second group was frozen and thawed in an identical manner. All muscles were then cooked in boiling water. Shear force data are summarized in Table 3.

Freezing and thawing a muscle before the normal onset of rigor mortis, which induces the very rapid "thaw rigor," also induces a highly significant increase in tough-

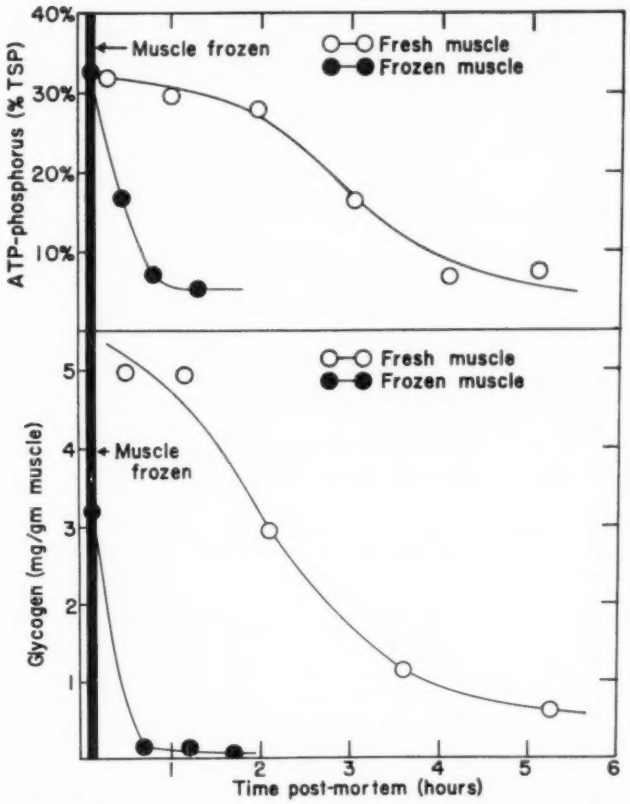

Figure 5. Effect of freezing and thawing fresh muscle on rates of ATP disappearance and glycogen breakdown.

ness of the cooked meat. The effect of freezing and thawing muscles which have passed through rigor mortis (second group) is negligible. Thus, the toughening effect of pre-rigor freezing is due, apparently, to the accelerated onset of rigor mortis.

Pool *et al. (24)* have presented data showing that whole, eviscerated birds which have been frozen rapidly post-mortem undergo considerable tenderization if they are held in the thawed state several hours before cooking. Although the data of Table 3 show that pre-rigor freezing and thawing results in muscle less tender than non-frozen muscle, there may still be significant, though incomplete, tenderization occurring in the thawed state. To test this point, 6 cockerels were killed and their *Pectoralis major* muscles were removed and bagged as described before. All muscles were frozen rapidly in a dry ice–ethanol bath and thawed immediately in running tap water. One muscle from each bird was cooked in boiling water, while their pairs were aged 24 hr at 2° C before cooking. In every case, the aged muscles were more tender than their pairs. The mean shear value of the unaged samples was 19.6 lb, while the aged samples averaged 7.7 lb. The probability that this result was obtained through sampling error was less than 0.005. These experiments show that the rapid rigor caused by freezing and thawing in the pre-rigor state causes a significant increase in toughness. This increase, however, can be partially reversed by aging 24 hr in the thawed state.

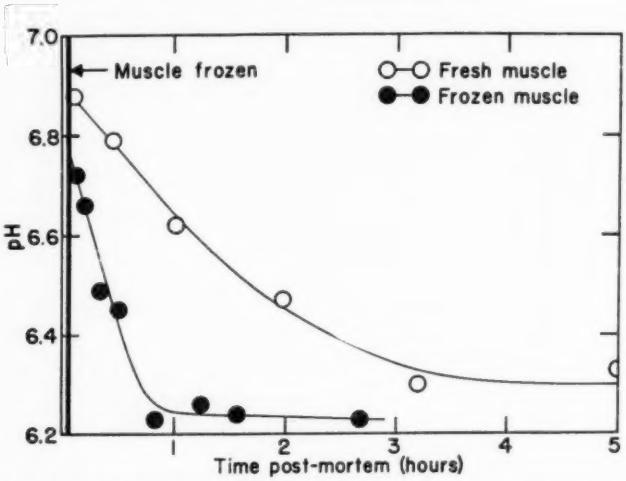

Figure 6. Effect of freezing and thawing fresh muscle on rate of decrease of pH.

Electrical stimulation. A study was made of the effect of exhaustive electrical stimulation, which depletes the glycogen reserve, on tenderness of muscle and the chemical changes of rigor mortis. A chicken was killed by bleeding and both *Pectoralis major* muscles were removed immediately. One muscle was stimulated intermittently (one-second intervals) with 90-volt DC shocks of 0.1 sec duration in a water-saturated nitrogen atmosphere until it no longer twitched visibly. The voltage was increased to 300 volts and stimulation continued until the muscle no longer responded. The total stimulation period was 15 min. Both muscles were placed in polyethylene bags and held in tap water (13° to 14° C). They were sampled periodically for ATP and pH. As expected, the rates of ATP disappearance and pH decrease were increased significantly in the muscle which had been electrically stimulated to exhaustion (Figure 7). A similar treatment was given to muscles from six 11-week-old chickens in order to determine the effect of exhaustive stimulation on tenderness. One *Pectoralis major* muscle from each bird was shocked intermittently with 20 volts DC in a water-saturated nitrogen atmosphere. The shocks were administered at one-second intervals and were 0.1 sec in duration. The voltage was gradually increased to a maximum of 390 volts and kept at this value until the muscles no longer responded. The total stimulation period was 30 min. The muscles were placed in individual polyethylene bags and stored at 2° C for a total post-mortem aging period of 24 hr before cooking. The mean shear value of the stimulated muscles was 12.0 lb and that for the control muscles was 8.5 lb. In every case, the shear value of the stimulated muscle was higher than that of its control. The probability that the mean difference was due to sampling error

TABLE 3

Effect of freezing and thawing cycle on muscle toughness

Aging time		Mean shear value		Mean pair difference [1]	P [2]
Before freezing	Total	Frozen-thawed	Control		
hr	*hr*	*lb*	*lb*	*lb*	
0.3	24	15.5	7.9	+7.6	<0.005
23	24	7.9	8.9	−1.0	>0.1

[1] Each value represents the mean difference of the 11 or 12 muscle pairs, the value for the control being subtracted from that for the frozen-thawed.
[2] Probability that the mean pair difference is due to sampling error.

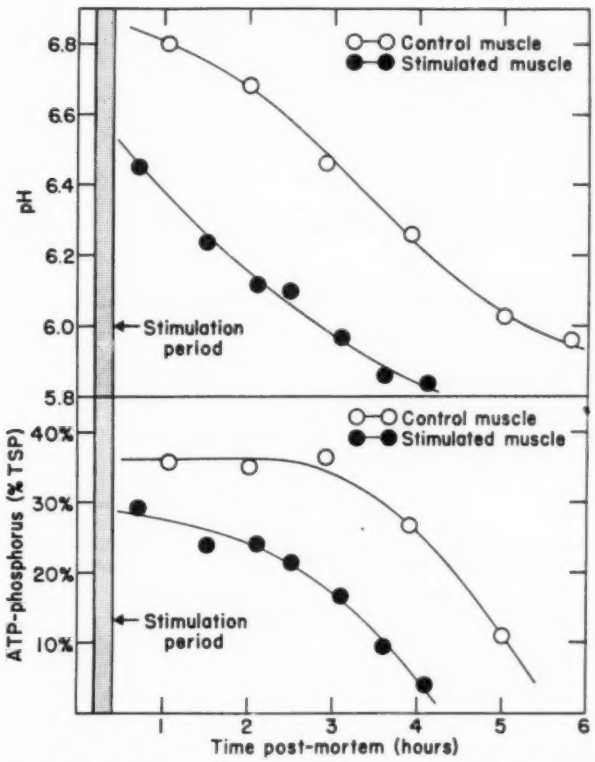

Figure 7. Effect of exhaustive electrical stimulation on rate of change of pH and ATP in paired muscles.

alone was less than 0.02. These results are in accord with the hypothesis that hastening the onset of rigor mortis makes the cooked meat less tender.

Electron irradiation. B. E. Proctor (private communication) has observed that chicken irradiated before having passed through rigor mortis is tougher in texture than samples irradiated after rigor. The effect of electron irradiation on muscle toughness was investigated by subjecting muscles to radiation from a linear accelerator. *Pectoralis major* muscles from each of 6 birds were removed rapidly, sealed in individual polyethylene bags, and aged for 24 hr at 2° C. Two additional groups of 6 chickens each were killed and the *Pectoralis major* muscles were removed and bagged as described above. One muscle from each of the 18 birds was irradiated with 2×10^6 rads in a linear accelerator (carried out by the Applied Radiation Corp., Walnut Creek, California). One of the freshly killed groups was allowed to age 24 hr at 2° C before cooking, while the other two groups were cooked and sheared as soon as practicable.

Muscles which were irradiated before the onset of rigor mortis were significantly less tender than their unirradiated controls (Table 4). Although no chemical determinations were performed on these samples, tactile observation showed that those muscles which had been irradiated while still fresh had definitely passed into rigor mortis during irradiation while their controls had not. This experiment supports the hypothesis that treatments which accelerate the onset of rigor mortis inhibit tenderization.

TABLE 4

Effect of electron irradiation on muscle toughness

Aging time		Mean shear value		Mean pair difference [1]	P [2]
Pre-irradiation	Total	Irradiated	Control		
hr	*hr*	*lb*	*lb*	*lb*	
0.5	2.8	32.0	17.0	+15.0	<0.005
0.5	27.1	30.1	11.5	+18.6	<0.001
25.0	28.0	13.6	9.2	+ 4.4	>0.2

[1] Each value represents the mean difference of the 6 muscle pairs, the value for the control being subtracted from that for the irradiated.
[2] Probability that the mean pair difference is due to sampling error.

Lethal injection of sodium monobromoacetate. "Alkaline rigor," or rigor mortis without concomitant acid production, has been known for many years. One method of producing this state is to kill the experimental animal by administering a lethal dose of insulin *(1, 3, 4, 5)*. When the animal dies, the muscle glycogen has been depleted and there is little or no acid production. This effect could not be tested in chickens since they are unusually resistant to the action of mammalian insulin *(27)*. Another method that we have used is to inhibit glycolysis by administration of sodium monobromoacetate. ATP cannot be regenerated anaerobically without glycolysis and the muscle passes very quickly into rigor mortis without production of acid.

Two groups of 11-week-old cockerels were starved overnight, stunned by electric shock, bled with an outside neck cut for 2 min, scalded at 60° C (140° F) for 30 sec, picked by hand, eviscerated immediately, chilled for 3.5 hr in ice slush, packaged in individual polyethylene bags, and frozen. The control and experimental groups were treated identically with the exception that the experimental birds received 150 mg of sodium monobromoacetate intravenously and were killed when death appeared imminent. After storage at −18° C (0° F) for less than 3 weeks, the birds were cooked from the frozen state in deep fat before evaluation of the shear value. The results showed that there was no difference between groups. This experiment was repeated with a 24-hr aging period and again there was no difference between groups with respect to shear value.

DISCUSSION

It has been firmly established by many investigators that the onset of rigor mortis occurs when the ATP level has dropped to one-half or one-third of its initial concentration. Our experiments have confirmed this in the case of chicken muscle, and also have indicated that the relative rate of onset of rigor mortis can be measured not only by following the disappearance of ATP but also by following the disappearance of glycogen and lowering of the pH. Another reaction, which we have not utilized but which does occur in aging muscle tissue, is the production of ammonia and inosinic acid from the adenine nucleotides. With one exception, all of the experiments that have been described in the preceding sections have shown that the more rapid the onset of rigor mortis (whether measured by breakdown of ATP or glycogen, drop of pH, or change in extensibility) the less tender will be the subsequently cooked meat. In the case of muscles which have been poisoned with sodium monobromoacetate, rigor mortis is characterized by a rapid breakdown of ATP but little or no change in glycogen and pH. The fact that these muscles are as tender as their untreated controls might seem to eliminate the rapid breakdown of ATP as the factor which induces muscle toughness. However, the ultimate pH of these muscles is appreciably higher than normal (pH 6.5 or greater, compared to normal values of 5.8

to 5.9). Since there is a positive correlation between pH (in the physiological range) and protein-bound water for beef *(29)*, it is possible that the monobromoacetate-poisoned muscles are tender solely because of the increased bound water of the tissue. Also, since the monobromoacetate may react directly with the muscle proteins, as Korman and Clarke *(15)* have shown with various other proteins in *in vitro* systems, this reaction might modify the muscle proteins in such a way that increased toughness would not develop. The monobromoacetate experiments do not provide conclusive evidence that increased toughness of muscles accompanying rapid onset of rigor mortis is caused by more rapid lowering of pH (or more rapid breakdown of glycogen).

There is no satisfactory theory to account for the marked increase in tenderness of meat during the post-rigor aging period. Older ideas crediting proteolysis for the phenomenon have been largely abandoned. Wierbicki *et al. (29)* have advanced the hypothesis that shifts in the relationships between proteins, water, and inorganic ions are responsible. Whether this or some other hypothesis is the ultimate answer, its mechanism must explain the puzzling circumstance that pre-rigor treatments which tend to accelerate the rate of onset of rigor mortis inhibit the development of optimum tenderness.

SUMMARY

The rate of development of rigor mortis and related biochemical changes in chicken muscle have been studied in relation to their effect on the ultimate tenderness of cooked muscle. Certain relationships, such as correlation between loss of adenosine triphosphate (ATP) and onset of rigor mortis, are qualitatively the same for chicken as have been reported for other species. In 10- to 16-week-old chicken, muscle at room temperature passes into rigor mortis 2 to 4½ hours post-mortem and reaches an ultimate pH of 5.8 to 5.9. Under the experimental conditions used, the initial ATP concentration averaged 4.8 mg/g muscle.

The physical changes associated with rigor mortis were followed by measurements of the relative extensibility of muscle strips. The effects of several variables on rate of loss of ATP (closely correlated with rate of onset of rigor mortis), rate of change of pH, and muscle toughness were investigated. These variables included excising the muscles pre-rigor, mechanical beating, post-mortem environmental temperature, freezing and thawing of fresh muscle, exhaustive electrical stimulation, electron irradiation, and lethal injections of sodium monobromoacetate.

Every treatment that resulted in more rapid loss of ATP (more rapid development of rigor mortis), more rapid drop of pH, and more rapid loss of glycogen also induced increased muscle toughness. Injection of sodium monobromoacetate, which causes rapid loss of ATP but only a small decrease in pH and glycogen, failed to induce toughness.

It is postulated that the relative toughness of cooked muscle in otherwise uniform groups of chickens increases with increasing rate of onset of rigor mortis or with some factor closely related to it.

Acknowledgments

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EFFECTS OF DIFFUSED LIGHT AND DARKNESS ON THE B-VITAMIN CONTENTS OF GERMINATING PULSES

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Marked changes in the vitamin contents of germinated seeds have been reported by several workers. An increase in the niacin content of soya bean, mung bean, lima bean, garden pea and green eye pea, during the early stages of germination, was observed by Katzkin *et al. (13)*. Burkholder and McVeigh *(4)* estimated thiamine, riboflavin, pyridoxin, niacin, inositol, pantothenic acid, folic acid, biotin and ascorbic acid in mature and sprouted seeds of *Pisum sativum*, *Phaseolus aureus* and seven varieties of *Sojax max*. While folic acid value decreased and thiamine value remained unchanged, pyridoxin, pantothenic acid, inositol, biotin showed slight increase in the germinated seeds. Cailleau *et al. (5)* observed in pea on the fourth day of germination much increased values of riboflavin and niacin and much decreased value of pantothenic acid. An increase during germination in the thiamine content of pea was reported by French *et al. (11)*. Nandi and Banerjee *(15, 16, 17)* observed increased values of ascorbic acid, niacin, and riboflavin in germinated pulses. Chattopadhyay *et al. (6, 7, 8, 9)* reported that thiamine, carotene, choline, and tocopherol contents of pulses increased during germination. Decreased values of folic acid and increased as well as decreased values of pantothenic acid of some pulses during germination were reported by Banerjee *et al. (1)*. As darkness is likely to affect the disposition of the vitamins in anotner way than in light, the effects of diffused light and darkness on the thiamine, riboflavin, nicotinic acid, pantothenic acid and folic acid contents of some common Indian pulses were investigated before and during germination for varying periods. Pantothenic and folic acids were estimated microbiologically and thiamine, riboflavin and nicotinic acid by fluorometric and chemical methods.

EXPERIMENTAL

About 5 g (1 g for pantothenic and folic acids) of healthy dry seeds of pulses was allowed to germinate in a petri dish for different periods up to 120 hr at room temperature (28–30° C) either in diffused light or in darkness. Different vitamins were estimated in the ungerminated seeds and in the seeds germinated for different periods and the vitamin values were expressed in terms of the original weight of the seeds before germination.

Estimation of thiamine. Powdered ungerminated seeds and seeds germinated for varying periods were crushed to smooth cream and heated in a conical flask with 75 ml of 0.1 N HCl at 100°C on water bath for 25–30 min with shaking every 5 min and cooled. The pH of the solution was adjusted to about 4.5 with 2.5 M sodium acetate using bromocresol green as an external indicator. Five ml of 0.1 M sodium acetate buffer of pH 4.5 was then added to the solution followed by 0.2 g of takadiastase and 0.2 g of papain. The contents in the flask were incubated at 37° C overnight with the addition of a few drops of toluene as preservative. After cooling the volume of the contents in the flask was made up to 100 ml. It was then centrifuged. Five ml of the clear digest was then shaken with water saturated iso-butanol and the aqueous layer was oxidised to thiochrome according to the method of Harris and Wang *(12)*. The fluorescence of the solution was measured by the 'Lumetron' photo fluorometer using primary and secondary B_1 filters.

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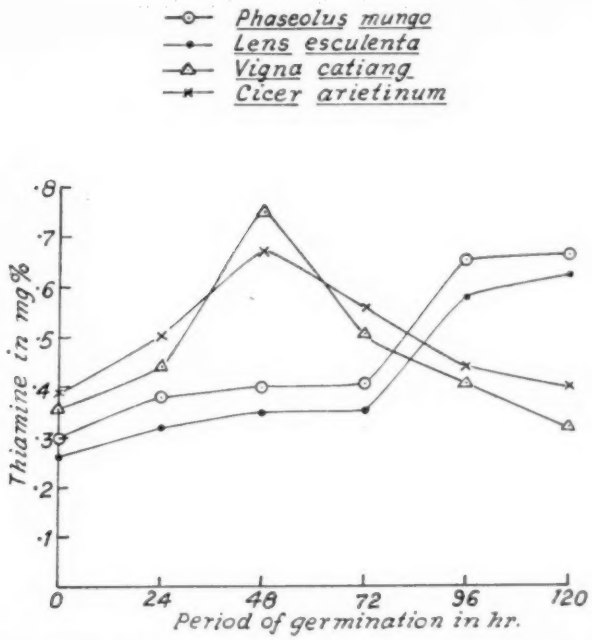

Figure 1. Effects of diffused light (L) and darkness (D) on the changes in thiamine content during germination of some leguminous seeds (absence of L and D on the graphs denotes similar effects).

Estimation of riboflavin. The method of extraction and digestion was the same up to the filtrate stage as in the case of thiamine, excepting that the heating in the boiling water bath was continued for 1 hr with shaking every 5 min. Fifty ml of the filtrate was then treated with 4% $KMnO_4$ and subsequently with 3% H_2O_2 for the removal of interfering non-specific fluorescent substances according to the method of A.V.C.A. *(14)*. The fluorescence of the solution was measured by the 'Lumetron' using primary and secondary B_2 filters.

Estimation of nicotinic acid. Ungerminated and germinated seeds were crushed to creamy stuff and heated with 100 ml of N HCl for 1 hr in a boiling water bath with occasional shaking. It was then cooled and the volume was made up to 100 ml and filtered. Fifty ml of the filtrate was taken in a beaker in a hot water bath and was treated with saturated solution of potassium permanganate and subsequently with a few crystals of disodium hydrogen phosphate and 40% NaOH to render the solution colourless according to the method of Banerjee *et al. (2)*. It was cooled, centrifuged, and adjusted to pH 6.6–6.8 using bromothymol blue as an external indicator. The volume was made up to 65 ml with water. To that 35 ml of 95% ethanol was added to make the total volume of 100 ml. The mixture was left in the refrigerator for 30 min and then centrifuged. The clear supernatant was used for the estimation of nicotinic acid by interaction with CNBr and *p*-aminobenzoic acid according to the method of Choudhuri *(10)*.

Estimation of folic acid. Ungerminated and germinated seeds were crushed in a glass mortar with 10 ml distilled water. Five ml of 0.1 M acetate buffer of pH 4.5 was added. The mixture was heated for 5 min in a water bath at 100° C. After cooling to room temperature 2 ml of hog kidney extract containing B_c conjugase, prepared according to the method of Bird, Robbins, Vanderbelt and Pfiffner *(3)*, was added to the mixture. After the addition of 0.5 ml of toluene it was incubated at 37° C for 24 hr. After

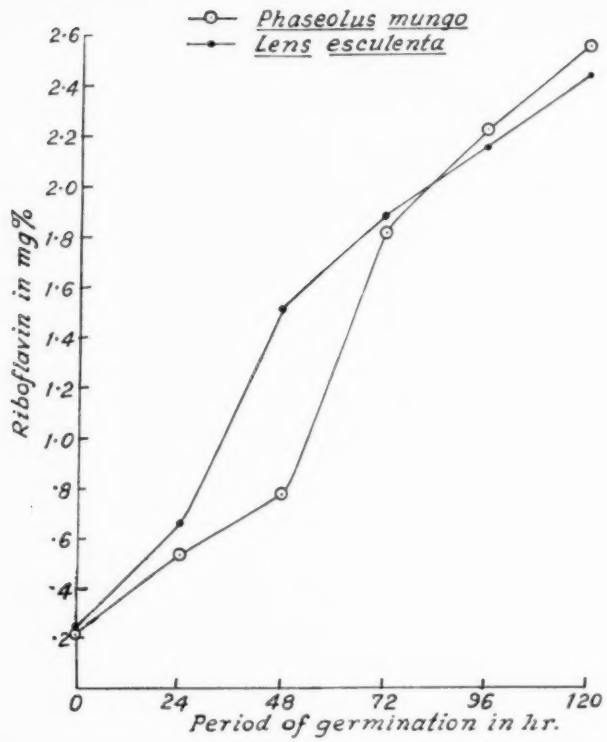

Figure 2. Effects of diffused light (L) and darkness (D) on the changes in riboflavin content during germination of some leguminous seeds.

incubation it was heated in a boiling water bath for 5 min, cooled and neutralised to pH 6.6 to 6.8 with 1 N NaOH. The volume of the extract was made up to 50 ml and filtered through Whatman No. 40 paper. The filtrate was diluted 10 times with water so that 1 ml of the diluted solution contained approximately 2 mmcg of folic acid. The folic acid in the extract was estimated using *Streptococcus faecalis R* (A.T.C.C. 8043) according to A.V.C.A. *(14)*. The growth of the microorganism after 18 hours' incubation at 37° C, was measured in the 'Klett Summerson' photoelectric colorimeter using 650 mmc filter. The folic acid content was calculated by interpolation from the standard curve and by subtraction of the enzyme blank from the value obtained.

Estimation of pantothenic acid. Ungerminated and germinated seeds were infused with 30–35 ml of boiling hot water in a conical flask for 20 min. It was then cooled and the pH was adjusted to 4.5 with 1 N NaOH. It was then made up to 50 ml and filtered through a fluted Whatman No. 40 paper. A suitable aliquot of the clear filtrate was adjusted to pH 6.6–6.8 with 1 N NaOH. It was then diluted 5 to 10 times, so that each ml of the diluted solution contained 0.05 mcg of pantothenic acid. Pantothenic acid was estimated turibidimetrically as in the case of folic acid using *Lactobacillus arabinosus* 17–5 according to the method of A.V.C.A. *(14)*.

All the vitamin values of germinating pulses in diffused light and darkness are shown in Table 1.

TABLE 1

B-vitamin contents of some Indian pulses during germination in diffused light and darkness. Results are expressed in mg per 100 g (those of folic acid in mcg per 100 g) and represent the average of 3 determinations. Local names are given in parentheses

Pulses	B-vitamins	Ungerminated	Effect of diffused light Period of germination in hr					Effect of darkness Period of germination in hr				
			24	48	72	96	120	24	48	72	96	120
Phaseolus mungo (kalai)	Thiamine	0.30	0.38	0.40	0.40	0.65	0.66	0.38	0.42	0.42	0.66	0.67
Lens esculenta (Musuri)	Thiamine	0.26	0.32	0.35	0.35	0.58	0.62	0.32	0.36	0.35	0.60	0.65
Vigna catiang (Barbati)	Thiamine	0.36	0.44	0.75	0.50	0.40	0.22	0.45	0.76	0.47	0.42	0.25
Cicer arietinum (Kabuli chhola)	Thiamine	0.39	0.50	0.67	0.56	0.44	0.40	0.50	0.67	0.56	0.44	0.42
Phaseolus mungo	Riboflavin	0.22	0.54	0.78	1.82	2.22	2.56	0.56	0.79	1.81	2.24	2.55
Lens esculenta	Riboflavin	0.25	0.66	1.52	1.88	2.16	2.44	0.66	1.53	1.87	2.17	2.44
Vigna catiang	Riboflavin	0.29	0.56	1.54	1.86	2.24	2.48	0.56	1.54	1.88	2.24	2.47
Cicer arietinum	Riboflavin	0.29	0.61	0.81	1.24	1.98	2.36	0.61	0.81	1.26	1.99	2.39
Phaseolus mungo	Nicotinic acid	1.3	1.7	1.9	2.1	2.5	3.6	1.7	1.9	2.2	3.6	4.1
Lens esculenta	Nicotinic acid	1.6	1.8	2.0	2.2	2.5	2.7	1.8	2.0	2.2	2.4	3.0
Vigna catiang	Nicotinic acid	1.2	1.6	1.7	1.9	2.6	2.7	1.5	1.8	2.2	2.7	2.9
Cicer arietinum	Nicotinic acid	1.5	1.5	1.6	1.8	2.3	2.5	1.5	1.6	1.8	2.3	2.5
Phaseolus mungo	Pantothenic acid	1.3	2.0	2.8	3.0	3.2	3.5	2.1	2.8	3.0	3.3	3.8
Lens esculenta	Pantothenic acid	1.2	1.2	1.1	1.1	1.1	1.1	1.2	1.1	1.1	1.1	1.2
Vigna catiang	Pantothenic acid	1.4	1.4	1.5	1.4	1.4	1.4	1.4	1.5	1.4	1.5	1.5
Cicer arietinum	Pantothenic acid	1.2	1.2	1.3	1.3	1.3	1.2	1.2	1.3	1.3	1.3	1.3
Phaseolus mungo	Folic acid	165	110	125	135	140	162	108	122	132	136	75
Lens esculenta	Folic acid	50	26	24	24	24	20	27	25	24	22	8
Vigna catiang	Folic acid	250	135	60	40	38	27	134	61	42	37	26
Cicer arietinum	Folic acid	200	56	52	46	28	16	57	53	45	26	18

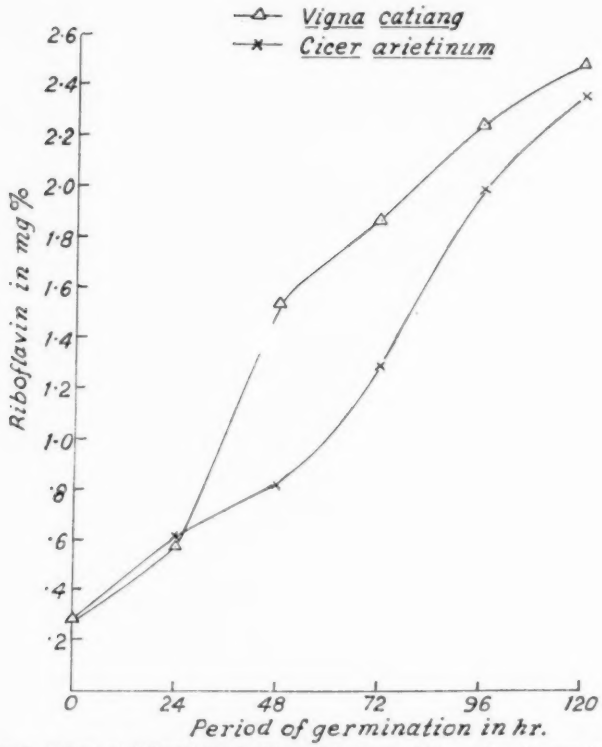

Figure 3. Effect of diffused light (L) and darkness (D) on the changes in riboflavin content during germination of some leguminous seeds.

RESULTS AND DISCUSSION

Effects of diffused light and darkness on the B-vitamin contents of some pulses during germination were found to be generally similar (Figs. 1–6). Thiamine content increased both in diffused light and darkness reaching a maximum value during 96–120 hr in *Phaseolus mungo, Lens esculenta* and during 24–48 hr in *Vigna catiang* and *Cicer arietinum*. Ribobavin and nicotinic acid increased greatly with germination both in diffused light and darkness in all the cases, but nicotinic acid was higher in darkness during 72–120 hr in *Phaseolus mungo*. Slightly higher values in darkness for nicotinic acid were also observed during 96–120 hr in *Lens esculenta* and *Vigna catiang*. Pantothenic acid increased considerably more in darkness during 96–120 hr only in *Phaseolus mungo*, while its value remained practically unaltered in all other cases during germination both in diffused light and darkness. Folic acid content decreased greatly with germination under diffused light in *Lens esculenta, Vigna catiang* and *Cicer arietinum*, but with *Phaseolus mungo* a fall was recorded during 24 hr, then there was a rise with the attainment of nearly the original value. With germination in darkness, however, the values

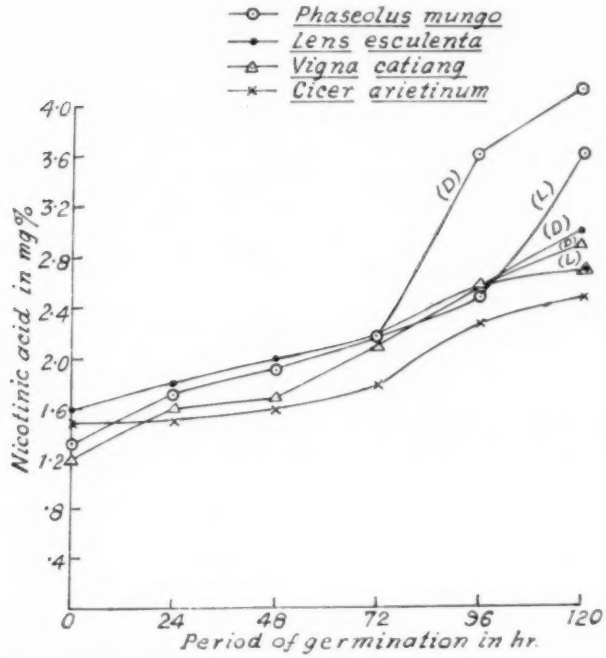

Figure 4. Effects of diffused light (L) and darkness (D) on the changes in nicotinic acid content during germination of some leguminous seeds.

fell off sharply towards the end in *Phaseolus mungo* and *Lens esculenta*, while they remained more or less the same as those in diffused light in *Vigna catiang* and *Cicer arietinum*.

The mechanism by which the concentration of some vitamins is augmented in the process of germination is unknown. It seems reasonable to suppose, however, that increased amounts of vitamins such as riboflavin and nicotinic acid which are known to be parts of important enzyme systems in intermediary metabolism might have a profound influence upon the supply of energy in growing tissues of seedlings. These pulses possess proteins of high biological value. Their important B-vitamin contents are increased to a large extent during germination. Hence these germinated pulses may be considered as a potential source of food which can easily be made available in large quantities.

SUMMARY

Thiamine, riboflavin, nicotinic acid, pantothenic acid and folic acid contents of four varieties of common Indian pulses were estimated before and during germination up to a period of 120 hours in diffused light and darkness. The first three vitamins were estimated by fluorometric and chemical methods and the last two microbiologically. Thiamine, riboflavin, nicotinic acid increased both in diffused light and darkness in all the cases with the progress of ger-

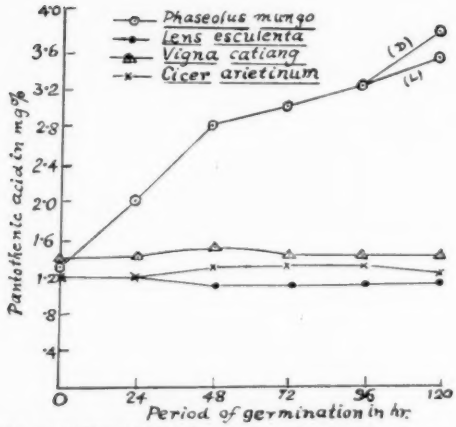

Figure 5. Effects of diffused light (L) and darkness (D) on the changes in pantothenic acid content during germination of some leguminous seeds.

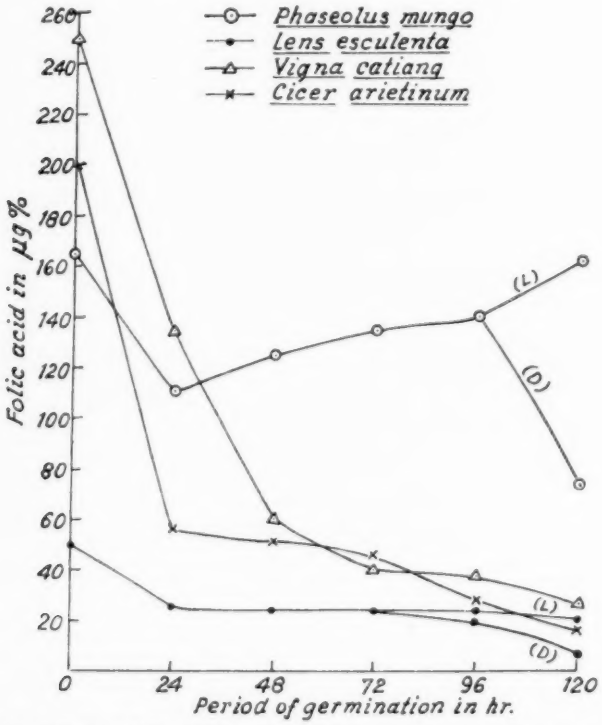

Figure 6. Effects of diffused light (L) and darkness (D) on the changes in folic acid acid content during germination of some leguminous seeds.

mination; the thiamine content, however, decreased after 48-hour germination in *Vigna catiang* and *Cicer arietinum*. The values of nicotinic acid in *Phaseolus mungo, Lens esculenta* and *Vigna catiang* were higher in darkness during the later period of germination. Pantothenic acid increased considerably both in diffused light and darkness in *Phaseolus mungo* only and its value was higher in darkness during 96–120 hours. Folic acid decreased greatly with germination, but with *Phaseolus mungo* the vitamin after a fall attained the original value. The folic acid contents fell off sharply in *Phaseolus mungo* and *Lens esculenta* towards the end of germination in darkness.

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THE DETERMINATION OF CHLORTETRACYCLINE IN TISSUES
I. AN IMPROVED PAD-PLATE METHOD

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Many studies have been reported on the marked effectiveness of chlortetracycline for the preservation of round fish *(3, 7, 8, 14–20)*. For the commercial use of chlortetracycline, however, government approval of the respective country is required. As an approach to realize this goal, it was necessary to make a survey of chlortetracycline residue in the flesh of fish which was subjected to various practical treatments with chlortetracycline. Tomiyama *et al. (21)*, Kline *et al. (12)* and Boyd *et al. (4)* have shown that the chlortetracycline content of the flesh of the treated fish is extremely minute and nearly completely destroyed by cooking or heat-processing the fish.

In order to set a reasonable chlortetracycline tolerance in fish it is essential to have a routine assay method which gives high sensitivity to and high recovery of minute amount of chlortetracycline in the flesh. The present paper deals with a proposal for an improved pad-plate method which meets the above requirements.

EXPERIMENTAL PROCEDURE

The proposed pad-plate assay method

1. Required apparatus, reagents and media.

a. Petri dish: Flat bottom dishes must be used. To prevent dew drops from falling on the medium it is safe to smear a water-repellent silicone over the inner surface of the cover.

b. Pad discs: Schleicher and Schuell No. 740-E; ¼ inch diameter.

c. Waring blender and incubator.

d. Extractant: A mixture of 40 parts of M/10 potassium citrate adjusted to pH 5.2 and 60 parts of acetone (by volume). The M/10 citrate solution can be prepared by mixing 100 parts of M/5 citric acid, 95 parts of N/2 potassium hydroxide and 5 parts of distilled water (by volume). For the preparation of chlortetracycline standard solution series, a citrate (pH 5.6)-acetone mixture of the same composition as the extractant is employed as a diluent of a stock chlortetracycline standard solution. The citrate solution having pH 5.6 is prepared by mixing equal parts of M/5 citric acid solution and N/2 potassium hydroxide solution (by volume).

e. Standard solution of chlortetracycline: A 2 ml aliquot of an 80.0 mg/100 ml water solution of crystalline chlortetracycline hydrochloride is placed in a small test tube, stoppered, frozen and stored in a freezer. To obtain a standard curve, the standard frozen CTC aliquot is diluted further with the diluent to provide solutions containing 0.16, 0.08, 0.04, 0.02, 0.01 and 0.005 mcg per ml.

f. Spore suspension: A spore suspension is prepared in a Roux flask using Medium No. 1 as given in Grove and Randall's manual *(11)*, except for a supplement of 50 mg $MnSO_4 \cdot 7H_2O$ per liter. The medium is inoculated with a saline suspension prepared from several slants of *B. cereus* No. 213 incubated 4 to 7 days at 37° C. The concentration of the inoculum must be adjusted so that it will show a light transmission of 5 to 20% at 660 mmc. The surface growth is suspended in 100 ml of sterile saline, centrifuged, and washed with sterile saline. The resulting sediment is suspended in 50 ml of sterile saline and heat shocked at 70° C for 30 min in a water bath, followed by immediate refrigeration.

g. Assay medium: The composition is the same as Medium No. 8 given in Grove and Randall's manual *(11)* except for the inclusion of 3 g monobasic potassium phosphate per liter and the addition of 1% equivalent of agar instead of 1.5%. Since the yeast extract was not available in our laboratory, a 3 g aliquot of a medicinal yeast ("ebios") was used. However, no difference was observed in the sensitivity of the test organism to chlortetracycline when the yeast extract was omitted. All the ingredients except agar are dissolved in 1 l of distilled water. The solution is heated, stirred with a 10 g portion of activated charcoal, followed by filtration. To the filtrate is added a 1% equivalent of agar and autoclaved for 10 min at 15 lb pressure. The pH value of this medium determined by the glass electrode should be 5.6–5.7.

2. Assay

a. The preparation of extract: A sample tissue is cut into small pieces and ground in a Waring blender. A 20 g aliquot of the homogenized sample is blended with 80 ml of the extractant for about 2 min, followed by filtration with dry filter paper.

b. The preparation of test plates: The cooled assay agar (about 55° C) is inoculated with a proper amount of *B. cereus* No. 213 spore suspension and a 5 ml aliquot is distributed evenly into each petri dish. These test plates are to be used within 2 hours.

c. Assay procedure: Dilute a stock standard solution (800 mcg per ml) with the extractant to the concentrations of 0.08, 0.04, 0.02, 0.01 and 0.005 mcg per ml. Three plates are employed for each concentration of the above standard solution series except the 0.02 mcg per ml concentration which is used as a reference. Three pad discs are dipped into one of the standard series, excessive amount of the solution being removed by touching to glass wall, and likewise three discs into the 0.02 mcg per ml reference. Place on each plate 3 standard discs and 3 reference discs, alternating standard and reference. Thus there will be 36 measurements of inhibitory zone diameters for the 0.02 mcg per ml and 9 measurements for each of the other concentrations of the standard solution series.

In the same way 3 plates are used for the sample extract. Place on each plate 3 sample discs and 3 reference discs, alternating sample and reference. After placing the cover of the dish, keep all the plates in refrigerator for 1 hr, and then incubate for about 16–18 hr at 30° C. Measure the diameter of each inhibitory zone.

d. The calculation of content: By employing the following formula, corrections can be made for each standard set for differences in time of placing the discs on the plate, in thickness of the medium, and in others:

$$S_i = s_i + (R - r_i)$$

where S_i = corrected value of the average of 9 zone sizes (mm) for standard solution at i mcg/ml.

s_i = average value of 9 zone sizes (mm) for standard solution at i mcg/ml.

R = average of 36 zone sizes (mm) for references placed on all the standard series.

r_i = average of 9 zone sizes (mm) for the reference placed on 3 standard plates for standard solution at i mcg/ml.

Draw the standard curve by plotting these corrected zone diameters, S, and the corresponding concentration of chlortetracycline (i, mcg/ml) on semilogarithm paper, the concentration being plotted on the logarithmic scale.

Corrected values of the zone sizes of the test sample sets can be similarly calculated.

From the standard curve read the concentration, A (mcg/ml) which corresponds to the corrected value of the zone size of the test sample set. Then, the antibiotic content of the test sample tissue can be calculated by the following formula: C (mcg/g tissue) = $A \times (80 + 20)/20$.

EXPERIMENTAL RESULTS

Effect of composition of the assay medium on sensitivity of the test organism to chlortetracycline. A comparative study was made between the Tomiyama *et al.* medium *(21)* and a Penassay base medium *(11)*. It was found that the sensitivity of the test organism to chlortetracycline was higher on the Penassay base medium than on the Tomiyama *et al.* medium which was rich in growth factors.

TABLE 1

The effect of pH of assay medium on sensitivity (zone size)

CTC concentration	pH of medium				
	5.3	5.5	5.7	5.9	6.2
mcg per ml	mm	mm	mm	mm	mm
0.2		24.4	22.7	21.7	20.0
0.1	(25)[1]	21.8	20.1	19.0	17.6
0.05	(22)[1]	19.1	17.4	16.1	15.2
0.025		16.2	14.5	13.4	12.7
0.0125		13.4	11.7	11.0	10.3
0.00625	(16)[1]	11.1	9.0	7.4	.....

[1] The growth of the test organism was so poor that no clear inhibitory zone was observed.

The sensitivity of the assay is much greater when the pH of the medium is low as shown in Table 1. However, in the pH range below 5.4, the growth of the test organism was so poor that a clear inhibitory zone could not be observed.

When the assay medium given above was used without adsorption treatment, the inhibitory zone did not usually show a sharp boundary below the 0.01 mcg/ml concentration and also tends to become smaller on extending the incubation time. However, this difficulty was eliminated by treating the medium with activated charcoal, and an improvement of the sensitivity in the lower concentration range resulted from this treatment. Furthermore, it was observed that the sensitivity in the lower concentration range was lowered when 1 mcg of thiamine, riboflavin, niacin, pantothenic acid, folic acid, p-aminobenzoic acid, biotin and 0.01 mcg of cobalamin were included in 1 ml of the charcoal-treated medium.

Dornbush *(6)* recommended the inclusion of potassium monobasic phosphate in the medium for improvement of the sensitivity. The effect of the concentration of the monobasic phosphate is presented in Table 2. It will be seen that the addition of 2.25 g of this phosphate resulted in yielding about 1.6 times as high sensitivity as the medium without the supplement.

Effect of thickness of the medium and strain on the test organism on sensitivity of the method. It is well known that thickness of the medium influences the zone size. The regular petri dish cannot provide a uniform thickness of the seed layer. The base layer placed under the seed layer is usually employed to make the seed layer of even thickness. A test was made to determine the change in sensitivity by using the same amount of base layer as that of seed layer. Data given in Table 3 indicate that the sensitivity was decreased by 25 to 35% when the double layer was employed. Furthermore, it was observed that the variations of zone size due to the uneven bottom surface was not appreciably eliminated by a double layer.

Grady and Williams *(10)* and others *(1, 12, 21)* employed *B. cereus* No. 5 as the test organism, whereas strain No. 213 was used by Tarr *et al. (16)* in the pad plate method. The latter strain is recommended for cylinder plate method in Grove and Randall's laboratory manual *(11)*. Hence, a comparison of the sensitivity to chlortetracycline in

TABLE 2

The effect of KH_2PO_4 concentration of medium on zone size (mm)

CTC concentration	KH_2PO_4 (g/L)				
	3.0	2.25	1.5	0.75	0
mcg per ml	mm	mm	mm	mm	mm
0.2	22.8	22.8	22.0	21.4	20.5
0.1	20.0	20.2	19.4	18.7	18.0
0.05	17.3	17.4	16.7	16.2	15.5
0.025	14.7	14.6	14.1	13.6	12.9
0.0125	12.0	12.1	11.6	11.2	10.7

TABLE 3

The effect of thickness of medium on zone size (mm)

CTC concentration [1]	Seed layer	Seed layer + base layer
mcg per ml	*mm*	*mm*
0.1	18.0	13.5
0.05	14.5	10.0
0.025	11.0	7.1
0.0125	8.2	....

[1] CTC was diluted with M/10 KH_2PO_4.

TABLE 4

A comporisan of sensitivity to CTC, as expressed in zone size, between B. cereus No 213 and No 5

CTC concentration	B. cereus	
	No 213	No 5
mcg per ml	*mm*	*mm*
0.1	21.9	16.9
0.05	19.0	14.4
0.025	16.0	11.7
0.0125	12.8	9.0

the pad plate method was made between these two strains. Data given in Table 4 demonstrate that strain No. 213 was about 2.8 times as sensitive as No. 5. Furthermore, the boundary of the inhibitory zone was clearer by using the former strain, No. 213, than the latter, No. 5.

Effect of refrigeration before incubation on the sensitivity. It is a general precaution that the pad plates should be refrigerated for one hour before incubation at 30° C *(1)*. A study was carried out to determine what effect holding the plates at about 5° C for one hour will exert on the sensitivity of the method. Data in Table 5 show that this treatment resulted in higher sensitivity than without it.

Effect of kinds of extractant on sensitivity. Table 6 shows a comparison between effects of using various kinds of extractants on the sensitivity of the method. Improvement in sensitivity was noted by using the citrate solution or citrate-acetone mixture and monobasic potassium phosphate solution as compared to an aqueous solution of chlortetracycline alone. It is to be noted that a hydrochloric acid-acetone solution (pH 4.2) of chlortetracycline gave higher sensitivity in the presence of the extractive substances of the flesh, whereas no such difference was found in case of the citrate-acetone solution (Table 7).

Effect of kinds of extractant on the recovery of chlortetracycline from tissues. Such extractants as a 0.57 N hydrochloric acid-acetone solution *(9, 10, 13)*, a hydrochloric acid-acetone solution adjusted to pH 4.2 *(1)* and an M/10 monobasic potassium phosphate

TABLE 5

The effect of refrigeration on zone size (mm)

CTC concentration	With 1 hr. refrigeration at about 5° C	Without refrigeration
mcg per ml	*mm*	*mm*
0.1	18.5	17.2
0.05	15.8	14.4
0.025	12.8	11.7
0.0125	9.9	8.9

TABLE 6

Difference in sensitivity of assay when CTC is dissolved in various extractants or flesh extract

Kinds of extractant	CTC conc (mcg per ml)				
	.1	.05	.025	.0125	.00625
	mm	mm	mm	mm	mm
Citrate acetone, pH 5.7	20.9	18.0	15.0	12.1	9.3
M/50 Citrate, pH 5.2	19.8	17.0	14.0	11.3	8.4
Flesh extract with HCl-acetone, pH 4.2	18.4	15.6	12.7	10.0	6.3[1]
M/10 KH_2PO_4	17.2	14.2	11.3	8.4	6.3[1]
Distilled water	15.6	12.6	9.7	7.7	6.3[1]

[1] Negative (no inhibitory zone).

TABLE 7

Difference in sensitivity between CTC solutions, in flesh extract and in extractant or diluent alone

Sample	CTC conc (mcg per ml)				
	.1	.05	.025	.0125	.00625
	mm	mm	mm	mm	mm
Acid-acetone, pH 4.2	15.9	13.0	10.0	8.3	7.0
Flesh-extract[1] with acid-acetone, pH 4.2	16.4	13.8	11.4	9.0	6.6
Citrate-acetone, pH 5.7	21.0	18.0	14.9	12.1	
Flesh-extract[1] with citrate-acetone, pH 5.2	20.8	17.9	15.0	12.2	

[1] Extract of horse mackerel.

solution *(11)* have been used for assay of chlortetracycline in tissues. Tomiyama *et al. (21)* reported that the recovery was about 85% when sample tissues containing the antibiotic was extracted with the 0.57 N hydrochloric acid-acetone solution. A comparative study on the efficacy of extraction between several extractants gave results which are presented in Tables 8 and 9. It is seen that the citrate-acetone mixture gave the highest recovery over the antibiotic concentration range of 0.1–0.5 ppm of the tissue. It is to be noticed that the recovery hardly varied with freshness of sample tissue. The next best extractant was the hydrochloric acid-acetone adjusted to pH 4.2, followed by the M/10 monobasic potassium phosphate solution. These results suggest that citrate ion plays a role of removing the antibiotic which is in chelation with metals in the tissue. Therefore, the effect was studied by the addition of sodium hexametaphosphate to the M/10 monobasic potassium phosphate solution, because hexametaphosphate is sparingly soluble in a 65% acetone solution. As seen from Table 9, a marked increase of the recovery resulted from the addition of hexametaphosphate.

Effect of interfering substances in sample flesh on the determination. It is known that chlortetracycline can be inactivated by heating or by an oxidizing agent. Since it is likely that sample flesh contains ferric ions, a study was made of the interfering action of ferric ion on the assay of chlortetracycline. Data presented in Table 10 indicate that the potassium citrate can eliminate the destructive action of ferric ion.

On the other hand, if sample tissue contains some other preservatives capable of inhibiting the growth of the test organism, the assay may possibly give some erroneous value. Therefore, the relative sensitivity of the test organism to various kinds of food preservatives was tested. It will be seen from Table 11 that no interference on the assay occurs due to the presence of these preservatives within the concentration ranges which are permitted for food additive use.

TABLE 8

Recovery of CTC from fish flesh

Species of fish	Citrate-acetone CTC mcg/ml			Acid acetone, pH 4.2 CTC mcg/ml		
	0.5	0.2	0.1	0.5	0.2	0.1
	%	%	%	%	%	%
Lizard fish [1]	94		95			
Sea eel [2]	93		90	86	80	
Horse mackerel [3]	89		89			
"Itoyoridai" [4]	94		91			
Croaker [5]	92		89	82		85
"Tai" [6]	94		99	83		
Mackerel [7]	94		94	69		
Fresh		91				
Spoiled		95				
"Konoshiro" [8]		98			76	
"Kidai" [9]				85		80

[1] *Saurida undosquamis.* [2] *Muraenosox cinereus.* [3] *Trachurus japonicus.* [4] *Nemepterus virgatus.* [5] *Argyrosomus argentatus.* [6] *Chrysophrys major.* [7] *Scomber japonicus.* [8] *Clupanodon punctatus.* [9] *Taius tumifrons.*

TABLE 9

Recovery of CTC from fish flesh using various extractants

Extractant	Species of fish	CTC conc.	Recovery
		mcg per ml	%
M/10 KH_2PO_4	Croaker [1]	0.5	72
M/10 KH_2PO_4	"Konoshiro" [2]	0.2	68
M/10 KH_2PO_4 + 200 ppm HMP*	"Konoshiro" [2]	0.2	82
M/10 Citrate	Mackerel [3]	0.2	89

[1] *Argyrosomus argentatus.* [2] *Clupanodon punctatus.* [3] *Scomber japonicus.*
* Sodium hexametaphosphate.

TABLE 10

The effect of ferric ion on stability of CTC with or without citrate

Initial conc of CTC	Fe^{+++}	K-Citrate	CTC conc after 24 hr at 30° C
mcg per ml	*mcg per ml*		*mcg per ml*
5.0	0.5	0	3.1
5.0	0.5	M/50	4.2
5.0	0	0	4.5

TABLE 11

The relative sensitivity of several food preservatives in the present assay method

Food preservatives	Minimum inhibitory concentration	Relative sensitivity [1]
	mcg/ml	*mcg/ml*
Chlortetracycline	0.005	0.1
5-Nitro-2-furfuralsemicarbazone	0.3	20
Dehydroacetic acid	1,000	4,000
Butyl-p-hydroxybenzoate	200	3,000
Sodium borate	1,000	2,000
2,4-Hexadienoic acid	1,000	2,000

[1] Concentration required to yield a comparable zone size with that of 0.1 mg CTC per ml.

DISCUSSION

For the determination of a minute concentration of chlortetracycline as low as 0.005 mcg per ml, the only available methods are pad-plate or cylinder-plate assay methods which employ *B. cereus* spore-containing assay media. The pad-plate method which was first reported by Grady and Williams *(10)* is more appropriate for routine work. The method has been modified by Broquist and Kohler *(5)*, Tomiyama *et al. (21)*, and Abbey *et al. (1)*. The modifications consisted of the improvement in the media and the extractant. However, no study has been reported concerning the recovery of chlortetracycline from tissues except that of Tomiyama *et al. (21)*. Data in the present study clearly showed that the sensitivity of the method and the recovery of the antibiotic from the tissues depends mainly on the composition of the medium, nature of extractant, and strain of the test organism.

Data presented in Table 1 infer that the sensitivity of the test organism will be greater under conditions unfavorable to its growth. The higher sensitivity in lower concentration ranges of the antibiotic resulting from the charcoal treatment of the assay medium can be explained by removal of some growth factors in the medium. This explanation is evidenced by findings that riboflavin *(9)*, thiamine, niacin, and pantothenic acid *(13)* are capable of reducing the bacteriostatic potency of chlortetracycline. The favorable effect on the sensitivity caused by the inclusion of monobasic potassium phosphate in the medium may be accounted for partly by its buffer capacity and also presumably by a possible decrease in concentration of growth factor(s) required by the test organism due to a higher rate of its metabolism in the presence of this salt. In order to obtain a further increase of the susceptibility of the test organism to chlortetracycline, it might be of interest to attempt to reduce the biosynthesis of vitamins or growth factor(s) in the germinating cells by including some antimetabolite in the assay medium.

It was noticed that keeping the pad-plate in the refrigerator for about one hour before incubation affected the sensitivity of the method. This favorable effect of the cooling on the sensitivity may be due to inhibition of the germination of the spore while acetone vapor from the extract is vaporizing and also the extract may diffuse into the assay medium. It is to be mentioned that when the aqueous extract was employed, this refrigeration treatment did not give any beneficial effect on the formation of a sharp boundary of the inhibitory zone.

One of the most noteworthy improvements of this proposed pad-plate method was the high recovery of the antibiotic from the tissues. No reliable assay value can be obtained if the recovery is low and if it varies with nature of freshness and the chlortetracycline content of sample tissues. Tomiyama *et al. (21)* reported that the recovery value in their modified method employing a 0.57 N hydrochloric acid-acetone solution as extractant was not satisfactory. They used as the standard solution the extract of chlortetracycline-free tissue to which a known amount of chlortetracycline was added prior to extraction. This method of making the standard curve is rather tedious particularly when dealing with a large number of samples. However, a high recovery and no deleterious effect of the presence of tissue extractive substances on the assay sensitivity was accomplished by using potassium citrate

as the extractant, making it possible to use the standard solution which can be prepared by dilution with the citrate diluent alone.

The idea of adopting the potassium citrate as the extractant was based on a presumption that a part of the chlortetracycline in tissue might chelate with the protein through some metals, such as calcium, magnesium, iron, etc. If this assumption is correct, the other chelating agent such as hexametaphosphate actually makes the recovery higher when added to M/10 monobasic potassium phosphate.

It is of interest that when citrate was used as the extractant, the inactivation of chlortetracycline by ferric ion was completely eliminated. Bissett and Tarr *(2)* reported that chlortetracycline was more stable in tissues than in aqueous solution. Womack *et al. (23, 24)* demonstrated that a chlortetracycline protective fraction existed in egg yolk. On the other hand, Weiser *et al. (22)* maintained that chlortetracycline was less stable in meat than in saline. It is quite probable that there will exist pro- and anti-destructive ingredients for chlortetracycline in tissues. The citrate extractant undoubtedly plays some role in stabilizing the antibiotic included in the tissue during the extraction procedure. This stabilizing potency of the citrate may partly contribute to the high recovery of the antibiotic from the tissues.

A question arises whether the pad-plate method for chlortetracycline assay can be applied to the tissue containing other food preservatives. The present data make it clear that the method can be employed without error in the presence of the other food preservatives now in commercial use.

SUMMARY

The pad-plate assay method for chlortetracycline has been improved with respect to its sensitivity, accuracy and recovery of chlortetracycline from tissue. The modification has been made based on the following findings:

1. The sensitivity of the test organism to chlortetracycline and the accuracy of the method can be improved by adjusting the pH value of the medium to 5.6, including 3 g of monobasic potassium phosphate per liter of the medium, and further treating the medium with activated charcoal.

2. The test organism *B. cereus* No. 213 is about 2.8 times more sensitive than *B. cereus* No. 5.

3. The holding of the pad-plates in the refrigerator for one hour is effective in increasing the sensitivity when the extract contains high concentration of acetone.

4. When a mixture of potassium citrate (pH 5.6) and acetone is used as the extractant of chlortetracycline, higher recoveries can be obtained as compared with the other extractants. The recovery is not varied with kind, freshness, and chlortetracycline content of fish flesh.

5. The chlortetracycline when dissolved in the citrate-acetone mixture, gives nearly identical growth inhibition to *B. cereus* whether with or without the presence of extractive substances of tissues.

6. The chlortetracycline when dissolved in the citrate-acetone mixture, can be protected from deterioration due to ferric ion.

7. No food preservatives tested interfere with the pad-plate assay for chlortetracycline.

Acknowledgment

The authors are indebted to Mr. A. C. Dornbush of the Lederle Laboratories Division of American Cyanamid Company for kindly sending us a lyphilized specimen of *B. cereus* No 213. The work was supported in part by Japan Lederle, Ltd. and the American Cyanamid Company.

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THE DETERMINATION OF CHLORTETRACYCLINE IN TISSUES II. AN IMPROVED CYLINDER-PLATE METHOD

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Chlortetracycline in blood serum and urine can be determined easily by the cylinder-plate method, since no previous extraction of the antibiotic is necessary *(4)*. In the case of chlortetracycline-containing tissues, however, difficulties have been experienced in this laboratory in obtaining high recovery of chlortetracycline and also in preventing the aqueous sample extract from putrefaction during the incubation of the test plate.

The present study was undertaken to determine the best procedure for extracting chlortetracycline-containing tissues to obtain maximum recovery of the antibiotic, and also the conditions necessary for high sensitivity to the CTC by the cylinder-plate method.

EXPERIMENTAL PROCEDURE

The proposed cylinder-plate assay method

1. Extractant of tissue and diluent of stock chlortetracycline standard solution: As tissue extractant a mixture of 35 parts of M/10 citrate buffer of pH 5.2, 35 parts of acetone and 30 parts of distilled water by volume is used. Diluent which is employed to prepare chlortetracycline standard series has the same composition as the extractant except the M/10 citrate buffer is adjusted to pH 5.6.

2. Preparation of sample extract: Blend for 2 min a 20 g aliquot of homogenized sample tissue with 80 ml of the extractant. After standing for about 15 min, filter the mixture with dry filter paper.

3. Preparation of test plate: The composition of assay medium is the same as reported for the pad-plate method except the agar concentration is 1.5% instead of 1.0% *(6)*. Melt assay agar medium, cool to 55 to 60° C, and add the proper amount of spore suspension of *B. cereus* No. 213 as directed in the pad-plate assay *(6)*. After mixing, add 5 ml of the seeded medium to each petri plate and tilt the plates to spread the medium evenly over each bottom.

4. Assay procedure and calculation of content: Place the test plates on an appropriate rack so that the cover of the plate will be at a slight elevated position. This precaution helps acetone vapor escape from the plate. Dilute a stock chlortetracycline standard solution (800 mcg per ml) with the extractant to the concentrations of 0.04, 0.02, 0.01, 0.005 and 0.0025 mcg per ml. Three plates are employed for each concentration of the above standard solution series except the 0.01 mcg per ml conc which is used as a reference. Place 6 cylinders on each plate. Fill 3 cylinders with the 0.01 mcg per ml conc of chlortetracycline and 3 cylinders with one of the standard solution series, alternating reference and standard. Thus, there are 36 measurements of inhibitory zone diameters for the reference (0.01 mcg per ml) and 9 measurements for each of the other concentrations of the standard solution series.

Three plates are used for the sample extract. Place 6 cylinders on each plate. Fill 3 cylinders with the sample extract and the other 3 cylinders with the 0.01 mcg per ml conc, alternating sample and reference. Place covers on plates and incubate all plates for about 18 hours at 30° C. Measure the diameter of each inhibitory zone.

The calculation of chlortetracycline content can be made in the same way as previously reported *(6)* using the standard curve similar to that shown in Figure 1.

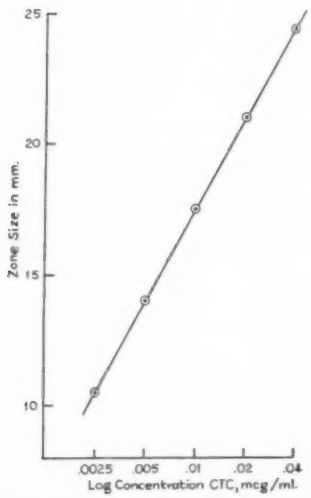

Figure 1. Standard curve for cylinder plate method for determining chlortetracycline.

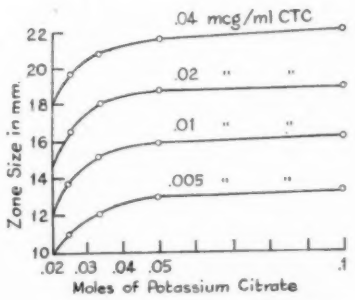

Figure 2. Effect of potassium citrate concentration on sensitivity (zone size).

RESULTS

Effect of pH and citrate concentration of extractant on sensitivity of method. The previous report *(6)* demonstrated that a citrate extractant was superior to M/10 monobasic potassium phosphate extractant in sensitivity for the pad-plate method. As presented in Table 1, a similar result was obtained in the present cylinder-plate method. The concentration of citrate influenced to a great extent the sensitivity of the method as illustrated in Figure 2.

As seen from Table 2, the sensitivity was also influenced by pH values of the citrate extractant. Below a pH of 5.2, the diluent was inhibitory to the growth of the organism. Above pH 5.2, the diluent gave no zones greater than the cylinder diameters (7.5–8.0 mm).

Effect of acetone concentration on sensitivity. Data presented in Table 3 indicate that the sensitivity of the test organism to chlortetracycline was apparently governed by the acetone concentration of the extractant. No blank value was obtained when the acetone concentration of the extractant was lower than 35% by volume. However, when the acetone concentration of the extractant was higher than 55%, the growth of the test organism on the plate was found to be strongly inhibited.

Interrelationship between concentrations of citrate and acetone on sensitivity of method. A preliminary trial showed that when tissues were extracted with extractants containing less than 35% acetone by volume, the extracts were found to contain protein. Hence, a study was made of the effect of citrate concentration of the extractant on the sensitivity of the method to acetone levels of 35% and 45%. It will be seen from Table 4 that variation of the citrate concentration in presence of acetone did not give as great an

TABLE 1

Effect of kinds of aqueous extractant on sensitivity (zone size in mm)

Extractants	CTC mcg/ml			
	0.04	0.02	0.01	0.005
	mm	*mm*	*mm*	*mm*
M/50 citrate, pH 5.7	20.0	17.3	14.1	11.2
M/10 KH_2PO_4[1]	17.5	14.9	12.5	11.1
Water	16.5	13.5	10.3	neg

[1] pH was adjusted to 5.7.

TABLE 2

Effect of pH of M/50 citrate on sensitivity

CTC	pH					
	5.0	5.2	5.5	5.7	6.0	6.5
mcg per ml	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
0.04	20.9	19.9	19.5	19.0	18.8	18.6
0.02	18.0	16.9	16.7	16.0	15.9	15.5
0.01	15.0	14.0	13.8	13.5	13.0	12.9
0.005	13.6	11.4	10.9	10.5	10.3	10.1
0	13.6	7.5	7.5	8.0	7.8	7.6

TABLE 3

Effect of acetone concentration of extractant [1] on sensitivity

CTC	% Acetone by volume				
	65 [2]	55 [2]	45	35	25
mcg per ml	*mm*	*mm*	*mm*	*mm*	*mm*
0.02	25.6	23.1	21.6	20.1	18.7
0.01	22.1	20.0	18.6	16.9	15.6
0.005	18.7	16.9	15.4	13.4	12.5
0	15.0	12.2	10.4	8.4	7.8

[1] The extractant consists of acetone x, water 65 — x, and M/30 citrate (pH 5.7), 35, by volume.
[2] No clear zone was observed due to poor growth of *B. cereus*.

TABLE 4

Change in sensitivity due to variation in molar concentration of citrate in presence of acetone

CTC	45% Acetone [1]			35% Acetone [2]		
	M/10	M/20	M/30	M/10	M/20	M/30
mcg per ml	mm	mm	mm	mm	mm	mm
0.02	21.9	21.9	21.6	20.5	20.6	19.8
0.01	19.0	18.7	18.7	17.3	17.4	16.9
0.005	17.0	15.5	15.8	14.6	14.6	14.1
0	12.4	8.5	7.8	8.0	8.2	7.0

[1] Consists of acetone, 45, water, 65-45, and each citrate solution, 35, by volume.
[2] Consists of acetone, 35, water, 65-35, and each citrate solution, 35, by volume.

effect on the sensitivity as in the absence of acetone. Since the pH value of the extractant is ordinarily shifted to higher pH value during extraction of sample tissues, a higher concentration of the citrate in the extractant is more appropriate than using a lower one in order to obtain the pH value of the extract around 5.6. In fact, when mackerel flesh was extracted with a mixture of acetone (35%), water (30%) and M/10 citrate buffers (35%) by volume having pH values of 4.8, 5.0, and 5.2, the extracts after removing acetone were found to give pH values of 5.55, 5.61 and 5.68, respectively. Based on the foregoing data, a mixture of 35 parts of acetone, 35 parts of M/10 potassium citrate buffer (pH 5.2) and 30 parts of water by volume is recommended as the extractant in the present method.

Further study was made to determine whether potassium ion aside from citrate ion plays a role in obtaining a higher sensitivity. Table 5 shows that potassium ion made a perceptible contribution to higher sensitivity.

Recovery of chlortetracycline from tissues by the present assay method as compared with other methods. It has been shown in the previous paper *(6)* that various types of extractants play an important role in the recovery of chlortetracycline from tissue. Data given in Table 6 show that the recovery of chlortetracycline included in horse mackerel

TABLE 5

Comparative effectiveness of sodium and postassium citrate [1] in obtaining high sensitivity

M/10 K citrate		35	25	17.5	10	0
M/10 Na citrate		0	10	17.5	25	35
		mm	mm	mm	mm	mm
CTC mcg/ml	0.02	21.4	20.7	20.4	20.1	20.1
	0.01	18.2	17.4	17.2	17.0	17.0
	0.005	15.0	14.4	14.2	14.0	14.0

[1] Citrate 35 + acetone 35 + water 30.

TABLE 6

Recovery of chlortetracycline by using various aqueous extractants

Extractants	CTC mcg/g		Recovery	Sample flesh
	Added	Recovered		
			%	
M/10 KH_2PO_4	0.200	0.150	75	Horse mackerel
M/50 K citrate	0.200	0.184	92	Horse mackerel
M/30 K citrate	0.200	0.180	90	Mackerel
0.1% Sodium hexametaphosphate	0.200	0.112	56	Horse mackerel
0.05% Sodium hexametaphosphate	0.200	0.117	59	Mackerel
0.02% Sodium hexametaphosphate	0.200	0.116	58	Mackerel

flesh was the highest when extracted with aqueous potassium citrate solution, next highest with monobasic potassium phosphate extractant, which is recommended by Grove and Randall's manual for an assay method of antibiotics *(4)*, and the lowest with a 0.1% sodium hexametaphosphate solution. On the other hand, when a citrate-acetone extractant was employed, an average recovery of 95% was obtained as given in Table 7.

It is to be noted that when monobasic potassium phosphate or sodium hexametaphosphate solution was used as an extractant, chlortetracycline included in the extract of horse mackerel flesh gave rise to a greater inhibition of the test organism than that included in these extractants alone (Table 8). When the acetone-citrate mixture was employed, a straight line relationship was held between zone size and logarithm of the concentration even at low concentration ranges down to 0.0025 mcg per ml; also the sensitivity of the test organism to the antibiotic gave small differences due to the presence of extractive substances in sample tissues as shown in Table 8.

TABLE 7

Recovery of chlortetracycline by using citrate-acetone extractant

Extractants	CTC mcg/g		Recovery	Sample flesh
	Added	Recovered		
			%	
45% acetone + M/20 citrate	0.200	0.201	100	Mackerel [1]
	0.200	0.176	88	Mackerel [1]
	0.200	0.194	97	Mackerel [1]
	0.200	0.181	90	Mackerel [1]
	0.200	0.184	92	"Kidai" [2]
35% acetone + M/10 citrate	0.040	0.0413	103	"Kidai" [2]
	0.080	0.0750	94	"Mebaru" [3]
	0.040	0.0399	100	"Mebaru" [3]
	0.080	0.0730	91	"Konoshiro" [4]
	0.040	0.0360	90	"Konoshiro" [4]

[1] *Scomber japonicus.* [2] *Chrysophrys major.* [3] *Sebastes inermis.* [4] *Clupanodon punctatus.*

TABLE 8

Change of sensitivity due to presence or absence of extractive substances of tissue

Acetone salt	Presence (+) or absence (−) of extractives	CTC mcg/ml			
		0.04	0.02	0.01	0.005
		mm	mm	mm	mm
45% K citrate	−	23.6	20.6	17.4	14.4
45% K citrate	+	23.7	20.5	17.5	14.0
35% K citrate	−	22.0	19.0	16.1	12.8
35% K citrate	+	21.7	18.9	15.8	12.9
0 M/10 KH_2PO_4 [1]	−	17.5	14.9	12.5	11.1
0 M/10 KH_2PO_4 [1]	+	18.0	15.7	13.5	11.4
0 0.1% NaHMP [2]	−	16.4	13.5	10.4	neg [3]
0 0.1% NaHMP [2]	+	17.1	14.5	11.9	neg [3]

[1] pH was adjusted to the same value as that with presence of extractive (pH 5.7).
[2] NaHMP = Sodium hexametaphosphate.
[3] Negative.

DISCUSSION

In Grove and Randall's manual for assay methods of antibiotics, a M/10 monobasic potassium phosphate solution is recommended as an extractant of sample tissue for the cylinder-plate method *(4)*. However, the previous report *(6)* indicated that the recovery was low when this extractant was employed in the pad-plate method. The present study also showed that low recovery and sensitivity resulted from using this extractant in the cylinder-

plate method. Another disadvantage of monobasic potassium phosphate as the extractant was that the sensitivity of the test organism to this phosphate solution of chlortetracycline varied with the presence of extractive substances in sample tissues (Table 8).

The lower recovery obtained by the use of sodium hexametaphosphate as the extractant was in contrast to the previous paper *(6)* which reported a favorable effect by the inclusion of hexametaphosphate into the phosphate extractant. This low recovery might be caused by an increased adsorption of chlortetracycline on the tissue protein which swells on addition of hexametaphosphate. This explanation is evidenced by a fact that the low recovery, 57%, was increased to 72% on coagulating the tissue protein in presence of hexametaphosphate by adding acetone at a 35% level.

In the pad disc-plate method, the acetone solutions containing hydrochloric acid *(1–3, 5)* have been used extensively to extract chlortetracycline in tissues. It was found in this study that the presence of acetone in the citrate extractant did not make any difference in the recovery of the antibiotic. Use of acetone in the extractant, however, was not only effective in obtaining protein-free extracts but also provided higher sensitivity of the assay. Since no blank value was noted below 35% level of acetone, the higher sensitivity seemed to be due to the higher diffusion rate of the acetone extract through the assay medium as compared with the aqueous extract. However, a higher concentration range of acetone was not appropriate for the determination, because the growth of the test organism was strongly inhibited during the incubation unless the acetone vapor is eliminated by some means. In the Yamazaki *et al.* modified cup method *(7)* use was made of a mixture of 35% dilute hydrochloric acid and 65% acetone as the extractant. It is easily seen from their standard curve that this method involves fairly high blank values due to both high acetone concentration and low pH value of the standard solution. It is apparent, therefore, that the poor reliability of the method, which is reflected by a considerable fluctuation in the recovery value, is due to the standard solution itself which is prepared by 65% acetone containing diluent without using tissue extract.

From the above consideration, it was decided to use the acetone (35%)-containing citrate solution as the extractant of chlortetracycline from sample tissues. This proposed method was found to give higher recovery and sensitivity than any other cylinder-plate method studied for determination of chlortetracycline in tissues.

SUMMARY

An improved cylinder-plate method for the determination of chlortetracycline in tissues has been presented. A mixture of acetone and citrate buffer has been employed for the extraction of chlortetracycline from sample tissues and also as the diluent of the standard chlortetracycline solution. The method provided a recovery of 95% in several kinds of fish flesh and a sensitivity of 0.002 mcg per ml.

Acknowledgment

The authors are indebted to Mr. A. C. Dornbush of the Lederle Laboratories Division of American Cyanamid Company for kindly sending us a lyophilized specimen of *B. cereus* No 213. The work was supported in part by Japan Lederle, Ltd. and the American Cyanamid Company.

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QUANTITATIVE DETERMINATION OF THE TRANQUILIZER DIQUEL IN ANIMAL TISSUES

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(Manuscript received February 3, 1959)

Tranquilizers as potential agents in anesthesia have been found useful for the past few years in veterinary practice. Chlorpromazine, among these drugs, has been successfully used as a basal anesthetic for operations in beef cattle *(7)* and dogs *(13)*, and as a belligerence reducing agent with horses *(10)*. Furthermore, because of its antiemetic properties and its beneficial effect on bronchial reflex irritability, chlorpromazine was found to be a better pre-anesthetic agent than morphine.

The use of chlorpromazine hydrochloride in newly weaned calves to diminish the shock of weaning *(8)* has been proved equally successful.

Similar action has been demonstrated *(14)* with Diquel (3-ethyl, 10-3′-dimethyl-amino-2′-methyl-propyl) (phenothiazine hydrochloride) (Jensen-Salsbery), the chemical structure of which is similar to that of chlorpromazine as shown in Figure 1. Analytical properties and method of determination for Diquel and chlorpromazine are analogous, both drugs belonging to the group of phenothiazine derivatives.

Among the various methods used to identify and differentiate this class of compounds, oxidation of the phenothiazine ring with the formation of colored compounds has been the one most widely adopted. For the colorimetric determination of phenothiazine drugs, either H_2SO_4 *(5)* or $NaNO_3$ in the presence of concentrated HCl *(9)* has been successfully used in the assay of organic materials *(1, 2)*. The spectrophotometric method of assay for phenothiazine derivatives has also been applied to estimation of chlorpromazine in body and tissue fluids with good results *(11)*. In all cases reported, however, the drugs were administered in relatively large doses (from 20 mg up to 100 mg and more per kg) and the sample size was small (about 4 g for tissues and 1–5 cc for fluids). Since the purpose of our study was to detect the presence of Diquel in organic material from large animals receiving 1 mg/kg, it was necessary to develop a procedure which would enable us to examine rather

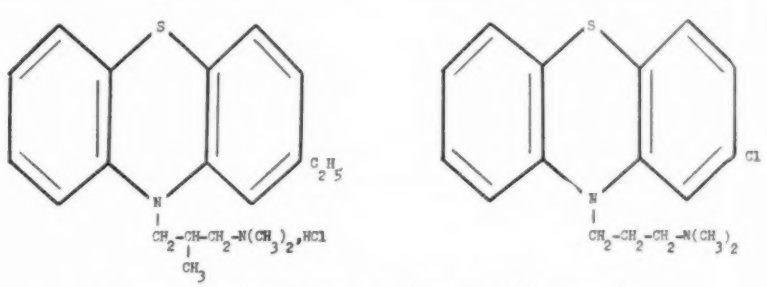

Figure 1. Structural formulas of Diquel and Chlorpromazine.

large amounts of tissues and body fluids and which was sufficiently sensitive to detect one part per million of the drug.

Although at the time we started our study no information was available as to concentration of phenothiazine drugs in large animal tissues following administration of therapeutic doses, the following facts seemed to be well established from the work of other authors *(2, 3, 5, 11)*:

1. A species difference exists as to concentration and rate of localization of phenothiazine derivatives in the different tissues.
2. The rate of disappearance from the various tissues is not the same for all phenothiazine derivatives and it varies from tissue to tissue.
3. The intensity of the drug's action and its duration are strictly related to drug concentration in various organs.
4. Chlorpromazine, when administered to rabbits by various routes is eliminated very slowly *(4)*.

We decided therefore to undertake a preliminary work with rabbits in order to test the rate of absorption of Diquel from the site of injection and to compare it with the results obtained by Berti and Cima *(4)*.

EXPERIMENTAL

In order to safely eliminate most of the bulk represented by protein material, the Berti-Cima method using a 1–3 mixture of ethyl alcohol and acetone was adopted, accomplishing Diquel extraction and protein precipitation in only one step. To the tissue extract evaporated to dryness in a water bath, 20% NaOH was added (1.5 cc for every 10 g of tissue). The sample was then kept for 1 hr on a water bath with occasional stirring to facilitate the saponification process. The semi-solid mass was then transferred to a

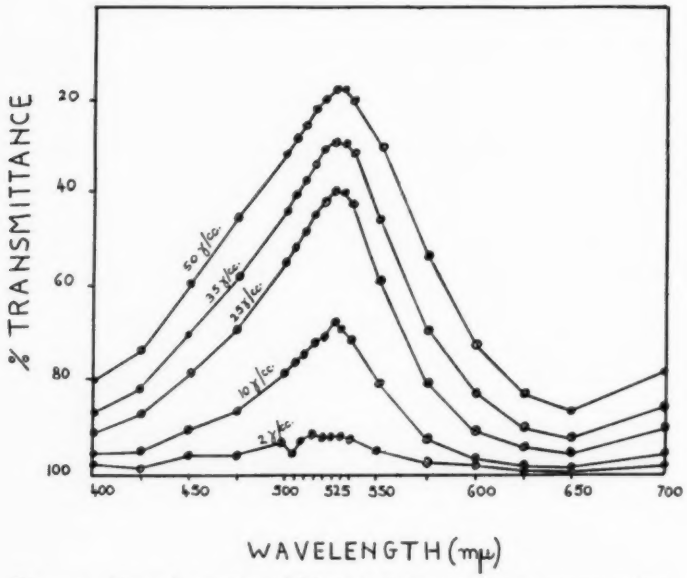

Figure 2. Absorption spectra of Diquel in H_2SO_4 at different concentrations.

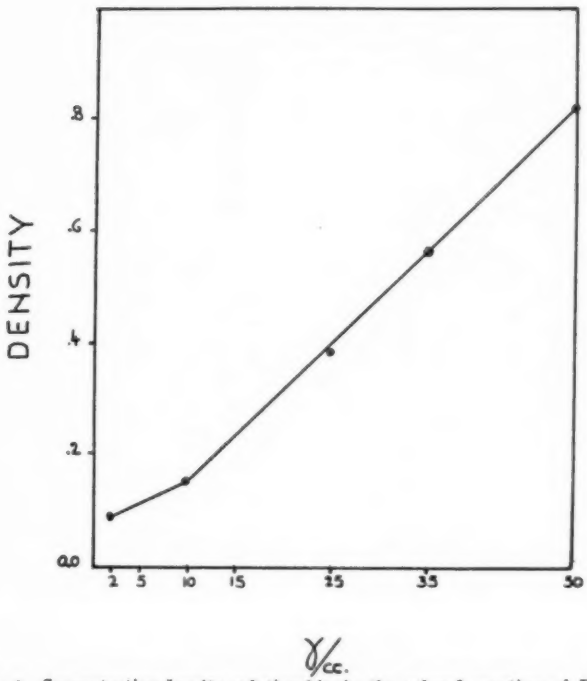

Figure 3. Concentration-density relationship in the colored reaction of Diquel with H_2SO_4 at 525 millimicrons.

Buchner (No. 3) and there, on filter paper (No. 42), washed three times with 20–25 cc of .5/N NaOH and once with distilled water. The solid mass, now free of glycerine and most of the pigment was then extracted 3 times with 30–35 cc of diethyl ether. The combined ether extracts (90–95 cc) transferred to a 150 cc separatory funnel, were washed once with 6 cc of .5/N NaOH and twice with 6 cc of distilled water. After an addition of a mixture of 3 cc of .1/N H_2SO_4 and .5 cc of H_2SO_4 (1:1), the ether extract was then shaken for 10 min at high speed.

Two and five-tenths cc of this acid-Diquel extract were finally mixed with 2.5 cc of concentrated H_2SO_4 according to the Dubost-Pascal method *(5)*. A blank and a standard were run simultaneously. Transmission values were read after 12 hr on a Bausch and Lomb spectronic 20 colorimeter at 525 mmc. The concentration of the unknown was calculated using the formula:

$$\text{Conc of Unknown} = \frac{\text{Density of the Unknown}}{\text{Density of the Standard}} \times \text{Conc of Standard}$$

The technique adopted for the color development was based on observations made in this laboratory during a preliminary spectrophotometric study of the reaction between concentrated H_2SO_4 and solutions of Diquel in .1/N H_2SO_4. When the percentage transmission values for solutions of Diquel containing from 2 to 50 gamma per cc were recorded at different wave lengths, the absorption spectrum of Figure 2 showing a peak absorption at 525 mmc was obtained. At this wave length concentration and optical density followed Beer's law satisfactorily from 10 to 50 gamma per cc (Figure 3), our findings indicating a different behavior of Diquel as compared to chlorpromazine. For the chlorinated derivative in fact, the absorption and concentration have been shown to be proportional from 2 to 15 gamma per cc *(1)*.

TABLE 1

Percentage recovery of Diquel from 28 g samples with 1 part to 10,000 of the drug added

Sample No.	Amount added	Amount recovered	Recovery
	mg	*mg*	%
1	2.75	1.944	
2	2.75	1.896	
3	2.75	1.944	
Average	2.75	1.93	70

The deviation from the Beer's law observed in the lower range seems to be confirmed by the absorption spectrum of Figure 2 where the curve referring to a solution with 2 gamma per cc departs from the pattern characteristic for higher concentrations.

The intensity of the color increased slowly until the final value was reached and the time required to attain such a point was between 7 and 8 hr. With different concentrations of Diquel it was also found that the lower the concentration the shorter the interval of time necessary to reach the final value. Figure 4 shows how at concentrations of 2 to 50 gamma per cc, transmittance values change after different lengths of time and how, with increasing concentrations, the difference between successive values widens.

Since it was possible to follow the color development for a maximum of 6 hr, the technique was then adopted of letting the color develop overnight at room temperature and reading the values the following morning. Once the final values were reached, transmittance remained unchanged when checked repeatedly over a period of almost 5 days.

The residue of Diquel at the site of injection was evaluated as follows: Three albino rabbits weighing 2.0 kg, 3.63 kg, and 2.73 kg. were administered the drug intramuscularly (1 mg/kg) and killed by bleeding after 8, 24, and 48 hr. The injection was given in the gluteal muscle by the following procedure: with the animals under light ether anesthesia the hair was removed from the posterior region over the gluteal muscle, the skin cut longitudinally with a sharp scalpel, the muscle exposed, and the needle (24 gauge) inserted parallel to the muscle fibers so that the entire dose was known to be in a well-defined area. The incision was closed with wound clips. Samples were taken for analysis by removing the entire muscle where the injection had been given. The average weight for each sample was 28 g.

A blank and triplicate sample for the standard were run at the same time. Since the doses given ranged from 2.5 mg to 3.75 mg, depending on the weight of the animal, the standard was prepared by adding 2.75 mg Diquel to 28 g of untreated muscle.

RESULTS AND DISCUSSION

By the colorimetric reaction with H_2SO_4, we are actually measuring Diquel simultaneously with those of its metabolites which maintain the phenothiazinic ring unchanged *(3)*. Since a recent chromatographic study of chlorpromazine metabolites has shown that the sulfoxide represents the major pathway of metabolism of these drugs in all species, the Dubost-Pascal method used in this work actually measures Diquel and its sulfoxide. However, because of the sedative action exerted by the sulfoxide metabolites on both dog and man *(12)* the estimation of Diquel and its sulfoxide derivative satsified the purpose of our investigation.

Tissue recovery. Using the method above reported, the recovery of Diquel at a concentration of 1:10,000 was 70% (Table 1).

However, since the main purpose of our investigation, on account of the low dose recommended for beef cattle (1 mg/kg) was to detect the presence of the least residual amount of the drug in muscle and soft tissues of beef cattle to be used for human consumption, a series of assays were run on muscle, liver and brain to study the percentage recovery attainable by our method at

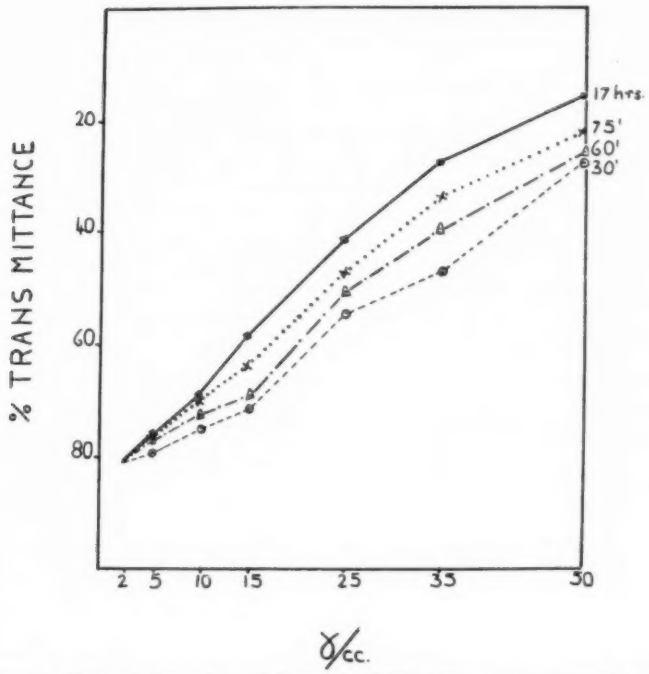

Figure 4. Transmission values of Diquel in H_2SO_4 with varying concentrations and varying time intervals (wavelength 525 millimicrons).

a much lower concentration and the influence of the sample size on the results. The chosen level was 1:1,000,000 (one gamma of Diquel per g of tissue).

Tissue samples for analysis, from 300–400 lb dairy calves killed by bleeding, were wrapped in aluminum foil, stored at −60° F and used within 10 days. At the time of analysis the corresponding amount of Diquel, from a 25 mg/cc aqueous solution of the drug, was added to the desired amount of tissue after thawing. Results obtained are presented in Table 2. Data reported would seem to indicate that the recovery varies from tissue to tissue, decreasing proportionately with increasing sample size. The relationship between per-

TABLE 2

Recovery of Diquel from different tissues of beef cattle at the level of 1:1,000,000 using samples of different size

Tissue	Sample size	Recovery
	g	%
Muscle	500	25
Liver	250	13
	150	28
	80	50
Brain	100	36
	80	45

centage recovery and amount of tissue analyzed, therefore, could be represented by the equation:

$$\text{Percentage recovery} = \frac{K}{\text{Sample size}}$$

where K is a constant characteristic of the tissue examined.

Absorption rate at the site of injection. When the percentage residue at the site of injection at different time intervals was determined by the technique previously described, the absorption rate appeared to be particularly slow. Data referring to the residue of the drug at the site of injection after 8, 24, and 48 hr are reported in Table 3. In the same table, the satisfactory agreement between the unknown concentrations as calculated from standard curve of Figure 3, using the 70% recovery above reported, and the values obtained using Beer's law, is also shown.

TABLE 3

Comparison between the percentage of residue as calculated from the density curve and the Beer's Law Formula in rabbits at different time intervals from an intramuscular injection of 1 mg/kg of Diquel

Rabbit No.	Time interval	Total gammas and percentage recovered as from curve		Total gammas and percentage recovered as from formula	
	hr		%		%
1	48	495	13	480	12.8
2	24	541	23	592	23.6
3	8	794	40	844	42.2

The slow process of absorption indicated by our results confirms the work of Berti and Cima *(3, 4)* pointing to a slow rate of elimination after intramuscular and subcutaneous administration.

While the results obtained with small and large animals demonstrate the sensitivity of the method, our findings, in agreement with the ones from the work of Berti and Cima would seem to anticipate the possibility of undesirable high concentrations of Diquel at the site of injection following intramuscular administration. Since this, in beef cattle for slaughter, would mean the loss of large pieces of meat not suitable for human consumption, it seems essential for future trials to use a new route of administration for a more rapid absorption and a more rapid elimination.

SUMMARY

The results of a spectrophotometric study on the colored reaction of Diquel with H_2SO_4 and the use of such reaction as a colorimetric method of determination of the drug in tissue are reported. The preliminary findings on the rate of absorption of the drug from the site of injection in rabbits and the necessity of exploring a new route of administration are discussed.

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SOME FACTORS CONTRIBUTING TO THE STABILITY OF FAT IN CHILLED DOUGHS[a,b]

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The growing demand for frozen prepared foods has drawn attention to those factors which limit the length of time initial quality is retained in these foods. One of the most important of these factors is the stability of the fat portion and much of the information presently available on frozen precooked foods deals with this problem. The addition of antioxidants to creamed turkey *(5)* markedly increased the length of time it could be stored frozen without becoming rancid. Precooked pork became rancid less quickly than raw pork *(9)*, due possibly to the destruction of lipoxidases in the meat. The presence of small amounts of soy flour in pastry retarded the development of peroxides during storage *(7, 8)*. The free fatty acid content of the pastry was low throughout the 12-month storage period. The peroxide content of stored frozen raw pastry was higher than when similar pastry was baked before storage *(8)*.

The stability of fats and fatty foods may be measured in several ways, the most rapid being the measurement of their peroxide and free fatty acid content. These products in the fat oxidation process are not always an accurate reflection of the condition of the fat since they themselves are constantly being broken down through further oxidation and other decomposition processes. Thus the quantities of these materials present may not always correlate with the results of Schaal oven tests which express the number of days necessary for the fat to become rancid at a temperature of 145° F, and those methods such as the Active Oxygen Method and the ASTM Oxygen Bomb Method *(3)* which are based on oxygen uptake by the fat. The Active Oxygen Method is applicable only to fats while the ASTM Oxygen Bomb Method may be used with foods.

MATERIALS AND METHODS

Preparation and storage of the pastries. Samples of prime steam lard and a commercially prepared hydrogenated vegetable shortening were used in this study. The following antioxidants and other additives, by weight percentage, were combined with the lard at 100° F and cooled to 72° F before use:

1. 0.01% BHA (butylated hydroxyanisole)
2. 0.01% BHT (butylated hydroxytoluene)
3. 0.003% citric acid
4. 0.003% propyl gallate
5. 0.05% commercial preparation A (20% BHA, 6% propyl gallate, 4% citric acid and 70% propylene glycol)
6. 0.05% commercial preparation B (20% BHT, 20% BHA, 30% oleo oil and 30% peanut oil)
7. 0.05% preparation B plus 0.003% propyl gallate

[a] Journal paper No. 174 from the American Meat Institute Foundation.

[b] Presented at the Nineteenth Annual Meeting of the Institute of Food Technologists, Philadelphia, Pennsylvania, May 18, 1959.

The doughs consisting of 58 parts flour, 26 parts fat, 1 part salt and 15 parts distilled water were mixed in the stainless steel bowl of a Hobart mixer. The flour and salt were mixed for 1 min; the fat added and mixed for 1 min; the bowl and beater were scraped and the mixing continued for an additional minute; the water was added with the mixer in operation and mixed for 1 min.

The dough was divided into 3 equal portions and sealed in 300 LSAT cellophane packages believed to be oxygen and moisture impermeable. Each portion of a single mixing was stored in a forced air freezer for the same length of time at one of 3 storage temperatures: 20°, 0°, or −10° F. The storage periods were 0, 2, 4, 6, 8, 10, 12, 20, 24 and 36 weeks. Following storage the doughs were allowed to thaw at 72° F before opening the packages. The dough was placed on a glass baking sheet, rolled with a glass rolling pin to ¼ inch thickness using 20 rolling motions, divided into 1½ by 2½ inch wafers and baked for 15 min in a gas oven preheated to 400° F. When cooled to 72° F the wafers from each storage temperature were distributed among 5 jars and placed in a Schaal oven at 145° F. Repeated tests indicated that the moisture content of the baked wafers fell between 5.4 and 5.8%. The samples were smelled daily until the wafers were judged rancid.

Analytical methods. The fat was extracted from the dough by blending a 90 g. sample with 150 ml of thiophene-free benzene for 5 min and filtering through a ½ inch layer of lightly-packed filter aid. An additional 100 ml of solvent was used to rinse the blender and filter bed. The filtrate was added to 30 g anhydrous sodium sulfate and placed in a refrigerator overnight. The dried filtrate was decanted into a 250 ml volumetric flask and benzene added to bring the extract up to volume. This procedure extracted 83% of the calculated fat in the dough and compared favorably with petroleum ether and a mixture containing 75% chloroform and 25% benzene, both of which extracted 82% of the fat.

These chemical tests were made in duplicate on 15 ml aliquots of the extracts: peroxide value, (A.O.C.S. Tentative method Cd 8-53), and free fatty acids, (A.O.C.S. Official method Ca 5a-40) *(1)*. In addition, carbonyl compounds were estimated on a small portion of the samples *(4)*.

The peroxide and free fatty acid content of the fat extracted from freshly made doughs was slightly greater than that of the test fat from which the doughs were made. The analyses of the dough extracts were used as control values throughout the experiment since they included the effect due to the flour lipids and the extraction procedure.

Carbonyl determinations were discontinued because of interference by the flour pigments present in the extracts. These pigments absorb in the same wavelengths as the 2,4-dinitrophenylhydrazones of the carbonyls and in addition were found to change in amount during storage.

RESULTS AND DISCUSSION

Effect of baking prior to storage. Two test pastries were made, one with lard containing commercial antioxidant preparation A, the other containing commercial antioxidant preparation B. Baked pastries, sealed in aluminum foil containers, were stored at 20°, 0° and −10° F and compared with pastries baked from the same lot of raw doughs which had been stored under similar conditions. Pastries were removed for Schaal oven tests at intervals up to 20 weeks. Pastries stored raw had a longer Schaal oven life than those baked before storage. Pastries containing preparation A became rancid after 36 and 31 days for raw and baked respectively, and those with preparation B after 161 and 150 days. Data analyses using Student's "t" test indicated a probable significance of 10% for the differences between raw and baked samples. Preparation B containing both BHA and BHT previously has been shown to be superior to BHA alone in carry through protection of pastries *(2)* and this seems to be true also in freezer storage. The greater stability of the fat phase in raw compared to baked pastry may be due to destruction of antioxidant *(6)* during baking resulting in a

lower concentration of antioxidant in the baked pastry during freezer storage. Fat hydrolysis during the first 20 weeks of low temperature storage appeared to be a minor contributor to fat instability, since the doughs frozen prior to heat treatment were more stable than those frozen later.

Effect of amount of fat. The standard formula used throughout the experiment had a fat-flour ratio of 44/100; this ratio is typical of pastries with a medium fat content. The ratio was increased to 60/100 to study the effect of a high fat formula. The pastries containing lard and 0.05% preparation A were stored in either the raw or baked state for 4, 8, 12, 16 and 20 weeks at 20°, 0° and −10° F. The mean Schaal oven life for the low and high formulae, 10 mixings each, were respectively 36 and 39 days for the pastries stored raw and 31 and 32 days for pastries stored baked. These differences were not great enough to be statistically significant when analyzed by Student's "t" test.

Effect of storage temperature. Commercially, most frozen pastry is marketed and stored before baking. The storage temperature may range from −20° F in commercial freezers to 20° F in the cold storage compartments of household refrigerators. The stability of 9 fats, incorporated in pastry dough, was followed during storage at −10°, 0° and 20° F for 2 to 36 weeks. The stability of the fats was assessed by the Schaal oven life of pastries baked from these doughs. The average stabilities for the entire storage period at each temperature are shown in Table 1. The stability

TABLE 1

Fat stability in pastries stored at three temperatures

Additive	Schaal oven life (days) [1]		
	Storage temperature		
	20° F	0° F	−10° F
None	32	28	30
Preparation A (1)	45	39	47
(2)[2]	33	33	34
Preparation B (1)	88	87	77
(2)[2]	153	154	146
Preparation B + propyl gallate	72	78	73
Propyl gallate	26	26	22
BHA	62	56	65
BHT	54	48	47
Citric acid	20	19	19
Hydrogenated vegetable shortening	72	76	73

[1] Mean value for 10 mixings of pastry per antioxidant.
[2] Separate experiment, represents 5 pastry mixings using another lard.

was similar at all three of the storage temperatures studied.

Stability during storage. The stability of the fat in raw pastry dough fluctuated during 36 weeks storage at temperatures of −10°, 0° and 20° F. Schaal oven life after 36 weeks storage was equal to, or only slightly less than the initial value. The pattern of stability fluctuation (Figure 1) varied with the different antioxidant preparations used. The pastry samples, except for those containing 0.01% BHA, increased in stability during the first weeks of storage. It is possible that this reflected the gradual equilibration of the antioxidants with the total fat system in the pastry.

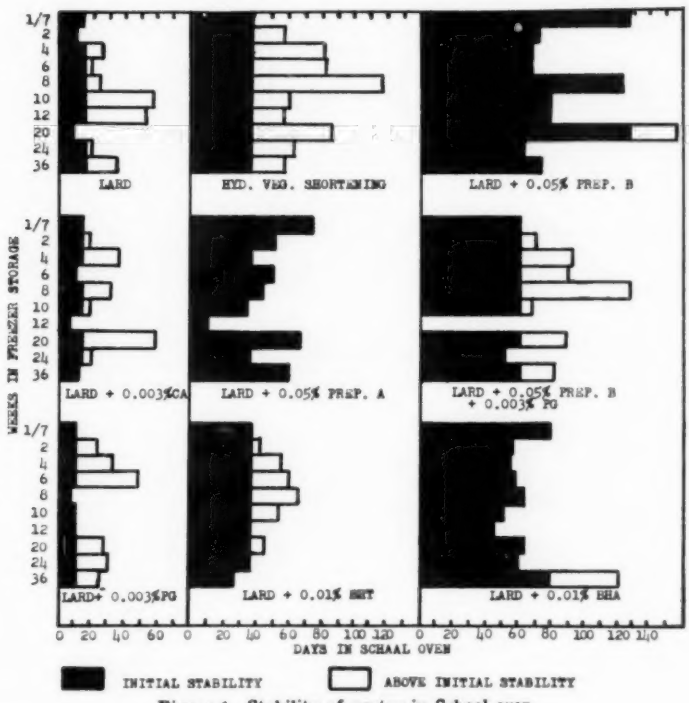

Figure 1. Stability of pastry in Schaal oven.

The stability of pastry made from prime steam lard increased very slightly while that containing hydrogenated vegetable shortening increased several times. This would support the above suggestion since hydrogenated vegetable shortenings are known to exert antioxidant properties through their tocopherol content. These too, would need to equilibrate with the naturally occurring lipids (about 1%) in the pastry flour. If this premise is correct, then it may be that BHA is either more rapidly distributed throughout the fat system or without effect upon the stability of the flour lipids.

A decrease in stability occurred at approximately 12 weeks of storage in all samples except those made from unstabilized lard. This was followed by an increase in Schaal oven life which reached its maximum at 20 weeks and then, except for those containing 0.01% BHA, decreased gradually during the remainder of the storage period (see also Figure 2). Samples containing 0.01% BHA decreased in stability from the peak observed at 20 weeks, but were either increasing or remaining constant in stability at 36 weeks. The same trends in stability were observed for −10°, 0° and 20° F storage.

The mean Schaal oven life for the three storage temperatures (15 jars) at each storage period are given in Figure 2, along with the range, illustrated by the shortest and longest Schaal life found among the 15 test jars.

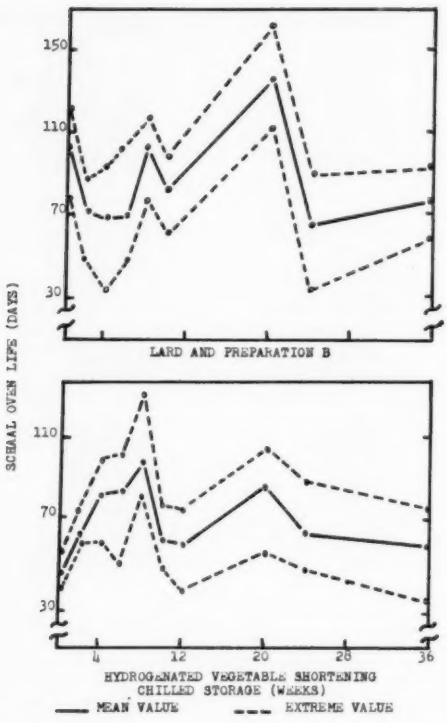

Figure 2. Mean and range of Schaal life of pastries at −10°, 0°, 20° F.

Pastries made with two representative fats are shown and the uniformity of the results from the 3 storage temperatures are apparent.

Relationship of Schaal oven stability to the development of fat oxidation. The change in amount of both peroxides and free fatty acids, during storage, was of low magnitude. The peroxide values ranged from 1.8 to 15.2 milliequivalents per kg of fat and the free fatty acids from 0.21 to 1.15%. Typical of the changes observed were the shifts in peroxide values in pastry made with lard stabilized with 0.01% BHT (Figure 3). The peroxides showed little relationship to Schaal oven life, reaching a maximum at 8 weeks' storage and decreasing to a low level in doughs stored more than 12 weeks. The concentration of free fatty acids in the pastry was so small that significant trends were not established.

Relationship of Schaal oven stability and fat oxidation changes measured by the ASTM bomb method, and development of peroxides and free fatty acids. The doughs used in this study contained prime steam lard and similar lard containing 0.05% preparation A and were stored at 0° and −10° F for 24 weeks. The Schaal oven stability of the doughs followed, throughout the storage period, the same trend as discussed above. Low peroxide and

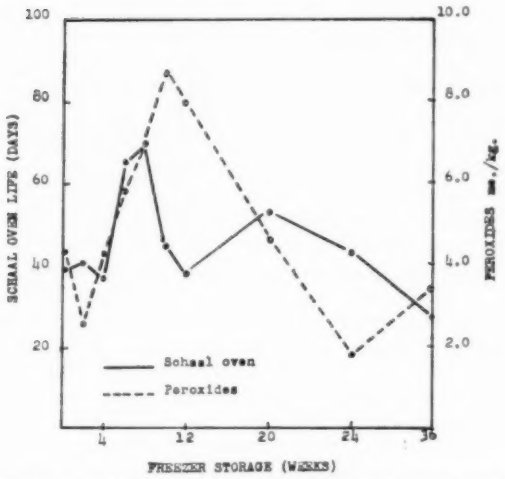

Figure 3. Schaal oven life and peroxide content of pastries made with lard and BHT stored at −10° F.

free fatty acid values also corroborated the previous findings. The stability of the fat content of the pastry, as indicated by Schaal oven storage, peroxide and free fatty acid content and oxygen uptake in the ASTM bomb, was the same during storage at 0° and −10° F. The ASTM bomb and Schaal oven data correlated closely (correlation coefficient 0.85) when values for pastry from both plain and stabilized lard which differed widely in stability were included (Table 2). This is similar to the correlation (0.81) reported for crackers *(3)*. However, the changes in fat stability during the storage period studied were so small that the correlation coefficients, for the fats calculated separately, were very low. This may be explained as previously suggested *(3)* by the inherent errors in the two methods.

SUMMARY

Pastry doughs were prepared using (1) prime steam lard and (2) similar lards containing, singly and in combination, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and citric acid.

TABLE 2

Schaal oven and ASTM oxygen bomb data for pastry stored at 0° and −10° F

Fat used	Storage temperature	Stability [1]		Correlation coefficient
		Schaal oven	ASTM bomb	
	°F	*days*	*hours*	
Lard	0	18.0	7.1	+0.62
	−10	16.0	7.3	+0.32
Lard + preparation A	0	54.0	17.8	+0.04
	−10	54.4	17.7	−0.17
All samples				+0.85

[1] Includes samples from 12 storage times.

The pastries were stored in either the raw or baked form at −10°, 0°, or 20° F for as long as 36 weeks.

The stability of the fat system in pastry was not affected by baking prior to low temperature storage, the percentage of fat in the pastry or the storage temperature between −10° F and 20° F. The addition of 0.003% citric acid or propyl gallate to the lard did not extend the Schaal oven life of the pastry made from it. The addition of 0.01% of either BHA or BHT markedly extended the fat stability of the pastry; a combination of BHA and BHT was even more effective. The stability of the fat varied throughout the 36 weeks storage period but there was no marked overall loss in stability. Increased stability was noted during the early part of the storage period and again at 20 weeks for all samples except those containing BHA. Those pastries containing BHA did not demonstrate the initial increased stability but did show a similar tendency to decrease in stability at 12 weeks and increase at 20 weeks. Peroxide and free fatty acid were present in such small concentrations that relationships to Schaal oven storage were indiscernable. Peroxide values increased slightly during the first 8 weeks of cold storage and thereafter remained at a low level. The ASTM bomb and Schaal oven stability data correlated well (0.85) for samples of diverse stability.

Acknowledgment

The authors wish to acknowledge the cooperation of Dr. B. N. Stuckey and Mr. Richard Sherwin, Eastman Chemical Products Inc., Kingsport, Tennessee in determining the ASTM bomb values used in this report.

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OBSERVATIONS ON THE BEHAVIOR OF MEAT PIGMENTS IN SOLUTIONS*

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A major problem in food technology today is the development of reliable objective tests which indicate changes in foods before deterioration is recognizable by organoleptic means. In meats, particularly beef, changes in acceptability are accompanied by a brownish discoloration. The color change is considered to be caused by conversion of the bright red pigment, oxymyoglobin (MbO_2), to the intermediary dark purplish pigment, reduced myoglobin (Mb), and then to the brown pigment, metmyoglobin (MMb). The reduced myoglobin pigment is found in negligible amounts on meat surfaces or in pigment solution since it is readily oxygenated to oxymyoglobin or oxidized to metmyoglobin.

Early work in this laboratory included a study of the behavior of meat pigment extracts, with an investigation of the possibility of developing an objective test for detecting meat deterioration by color change. The spectrophotometric method of Austin and Drabkin *(1)* modified by Jensen and Urbain *(6)*, which is routinely employed in meat color research, was used for the estimation of metmyoglobin in the presence of oxymyoglobin. This method for estimating the proportion of metmyoglobin in a mixture assumed to contain only two pigments, oxymyoglobin and metmyoglobin, is based on the fact that taken individually, at a standard concentration and pH, each of these two pigments will give a characteristic reproducible absorption curve. At any given wave length the difference between these two theoretical curves may be measured. This difference is considered to be the total change which would occur in a reading at a given wave length if oxymyoglobin were completely converted to metmyoglobin. The difference between the reading for oxymyoglobin and that for the unknown mixture at the chosen wave length is considered to represent the partial change the pigment has undergone. Three or four carefully chosen wave lengths which represent the maximum and minimum points of absorbance of oxymyoglobin are used in the estimation of the amount of metmyoglobin present. Estimates made at each of the wave lengths should be comparable and any differences noted should vary in a random manner. In the early studies, however, it was found that a systematic variation existed when the proportion of metmyoglobin was estimated at individual wave lengths. This was true whether the wave lengths 540, 560, 575, and 630 mmc recommended by Austin and Drabkin *(1)* for methemoglobin estimations or those of 544, 564, 582 mmc, recently reported by Bowen *(2)* for metmyoglobin were used. The estimates of metmyoglobin made at the wave length 544 and 582 mmc were less variable and consistently higher than those made at 562 mmc. When presumably identical solutions were used, estimates made at 582 mmc were inclosest agreement with each other *(8)*.

* Journal Series Paper No. 1975, approved by the Director, Missouri Agr. Exp. Sta.

When extinction coefficient ratios for mixed pigments were compared with those for pure pigment extracts at critical wave lengths, it was found that the ratios for 544/564 mmc did not fall between the ratios for the two pure pigment extracts as would be expected if the solution contained a mixture of only the two pigments *(8)*.

Both the failure to obtain comparable estimates of metmyoglobin formation at individual wave lengths and the anomalous ratios obtained at 544/564 mmc suggested the presence of a third pigment in the mixture. A compound described by Keilin *(7)* as resulting from treatment of metmyoglobin with hydrogen sulfide water has characteristics which could explain both systematic variation and anomalous ratios described above. This compound is a sulfmetmyoglobin analogous to cyanmetmyoglobin except that the sulfur is reversibly combined. Keilin contrasted this pigment with one described earlier by Hoppe-Seyler *(7)*. The Hoppe-Seyler pigment, which can be produced by bubbling hydrogen sulfide gas through myoglobin solutions in the presence of oxygen, is probably an irreversible ferrous derivative of myoglobin and some oxidation product of hydrogen sulfide. The Hoppe-Seyler pigment would not ordinarily be encountered in meat or meat extracts except as a result of bacterial action or specific chemical treatment.

Ginger, Lewis, and Schweigert *(5)* noted a cherry-red pigment occurring during irradiation of solutions containing high proportions of metmyoglobin. When the extracts contained high proportions of oxymyoglobin, they reported formation of a mixture of metmyoglobin and green pigment. The spectral curve of the cherry-red pigment was similar to that of oxymyoglobin but the evidence was not considered conclusive. There is a possibility that this cherry-red color might have been due to Keilin's pigment produced by combination of metmyoglobin with sulfur groups released during irradiation. The green color observed may have been due in part to formation of Hoppe-Seyler pigment.

Little other work which has been reported on the effect of sulfur compounds on meat pigments appears to have a direct bearing on the present problem.

The present investigations were undertaken to study the possibility of the presence of some naturally occurring sulfur-myoglobin pigment which might explain the systematic variation in the estimate of metmyoglobin at different wave lengths and the inconsistent extinction coefficient ratios in mixed pigment solutions. Various sulfur compounds were added to mixed and metmyoglobin pigment solutions in an attempt to exaggerate the noted systematic variation. Any evidence of such exaggeration might be considered to indicate the possibility that the added compound occurred naturally in small amounts in the pigment extract and might be responsible for the observed deviations from expected behavior.

EXPERIMENTAL PROCEDURE

Lean round of beef was obtained in several lots from a local store. The meat was twice ground in a large power meat grinder, then packaged in 1 lb portions, frozen and held in frozen storage until needed for experimental purposes.

For preparation of the pigment solution, enough one-fourth inch slices were removed from one end of the package to yield 50 g. These slices were placed in a Waring blender with 100 ml of boiled, cooled distilled water and blended for three minutes. The resulting

slurry was centrifuged 6 minutes. The fatty portion was lifted off the top and the remaining red liquid filtered in the refrigerator through fast filtering paper. The filtrate obtained was a clear sparkling liquid. Ten ml of the filtrate were diluted to 25 ml with boiled cooled distilled water for both treated and untreated solutions. This resulted in a pigment concentration of 0.045–0.046 mM per liter on the basis of porphyrin content. This was found to be a desirable concentration for absorption spectra readings.

A Cary recording spectrophotometer was employed for the determination of the absorption spectra of the solutions for the wave length range from 480 or 500 to 650 mmc. Three sets of control curves were obtained for each extract. The 3 control spectra were of the (a) untreated mixed pigment solution, (b) metmyoglobin pigment formed by treating the extract with a small potassium ferricyanide crystal, and (c) cyanmetmyoglobin obtained by adding one or two small crystals each, of potassium ferricyanide and potassium cyanide to the extract. These control curves were used for comparison with experimental curves obtained by treating the pigment solution with the different sulfur-containing compounds. The cyanmetmyoglobin curve was used for determination of the total porphyrin concentration in the solutions, since all the naturally occurring forms of myoglobin can be converted into this pigment. The millimolar concentration of pigment in the meat extracts was then calculated according to the method and formula given by Austin and Drabkin *(1)*. The millimolar concentration (c) is equal to the observed absorbance (E_o) of the solution at a specific wave length, divided by the millimolar extinction coefficient (ϵ) multiplied by the length of the light path (d) in centimeters.

$$c = \frac{E_o}{d\epsilon}$$

The chosen wave length for the determination of concentration of cyanmetmyoglobin was 540 mmc which is at the region of maximum absorbance for cyanmetmyoglobin. The millimolar extinction coefficient of 11.3 for cyanmetmyoglobin as given by Bowen *(2)* for this wave length was used.

When metmyoglobin with only a slight excess of potassium ferricyanide was used in experiments, it served as the control and concentration was determined as for the mixed pigment solutions.

The effects of the sulfur compounds, sodium hydrosulfite ($Na_2S_2O_4$), hydrogen sulfide gas, saturated hydrogen sulfide water, cysteine, glutathione, and 1-amino-2-naphthol-4-sulfonic acid were studied.

Sodium hydrosulfite, glutathione, and 1-amino-2-naphthol-4-sulfonic acid were added in the crystalline form to the mixed pigment and metmyoglobin solutions. Glutathione and 1-amino-2-naphthol-4-sulfonic acid had no apparent effect on the pigment solutions, and are not discussed further. Hydrogen sulfide gas was bubbled through the mixed pigment solution in order to form the Hoppe-Seyler sulfmyoglobin pigment. The formation of Keilin's sulfmetmyoglobin was studied by adding specified amounts of saturated hydrogen sulfide water to a metmyoglobin solution prepared by dialyzing the metmyoglobin solution prepared from the mixed pigment solution against boiled cooled distilled water for four days in the refrigerator. The water was changed daily. To study the effects of cysteine, a solution containing 2 mg of cysteine per ml was used. This was added in specified amounts to the metmyoglobin solution which contained only a very slight excess of potassium ferricyanide. The spectra of the solutions were read immediately after their preparation and thereafter at definite time intervals.

RESULTS AND DISCUSSION

In studying the behavior of the meat pigment, little variation was noted between the control curves for the different samples when the values were converted to millimolar concentration.

A study was made of the spectrophotometric characteristics of both Hoppe-Seyler and Keilin pigments which had been discussed by Keilin *(7)* in the earlier literature. This was done in order to recognize the characteristics of the spectrophotometric curves of these pigments and for purposes of comparison of mixed pigment solutions and sulfur treated solutions with these

recognized sulfur-myoglobin pigments. It was considered important to know whether or not Hoppe-Seyler pigments were present under any of the conditions of the experiments and whether or not characteristics in common with Keilin's pigment were obtained by the various treatments.

In the presence of oxygen, the addition of hydrogen sulfide gas to the mixed pigment solution resulted in the formation of the green Hoppe-Seyler pigment. The absorption curves for this pigment (Figure 1) showed a decided peak at 618 mmc, which is in agreement with the earlier findings. In addition to this, two minor peaks were noted at 545 and 578 mmc in the present study. Keilin *(7)* has explained the production of the green pigment as an apparently involved irreversible reaction between a ferrous compound and some oxidation product of hydrogen sulfide. He supported his theory by demonstrating that the addition of potassium ferricyanide to the solution destroyed the green pigment and eliminated the peak at 618 mmc. The addition of sodium hyposulfite to the potassium ferricyanide treated pigment caused the peak at 618 mmc to reappear. Thus, the green pigment was capable of undergoing oxidation so must have been a ferrous compound.

In the present experiments the addition of potassium ferricyanide to the green pigment destroyed the peak at 618 mmc and the addition of potassium cyanide produced little further change in the absorption curve (Figure 1).

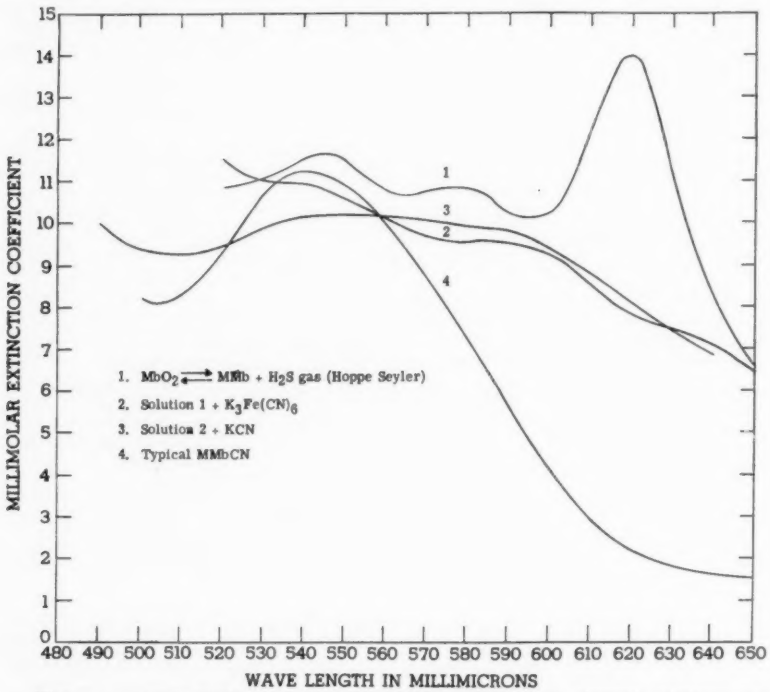

Figure 1. Spectrophotometric curves obtained when mixed pigment solutions are treated with H_2S gas.

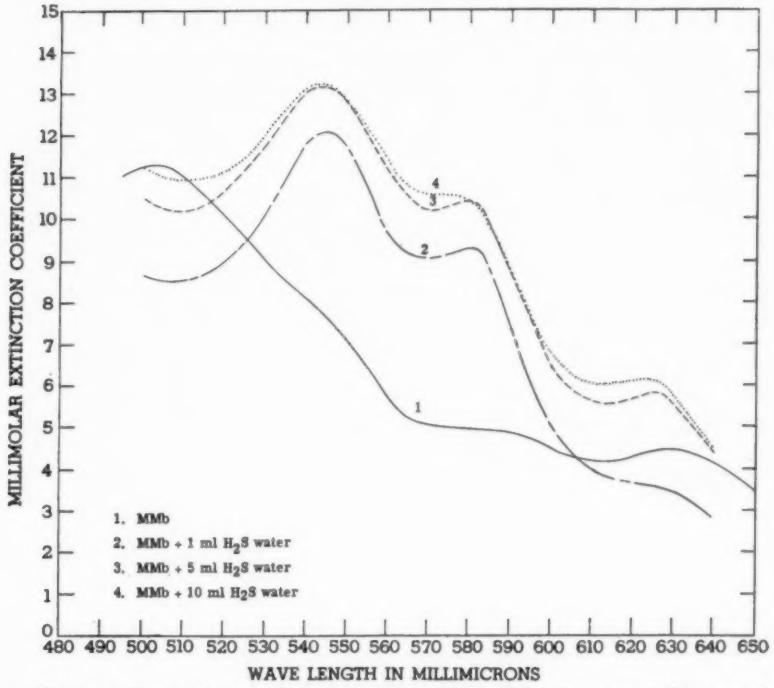
MILLIMOLAR EXTINCTION COEFFICIENT
WAVE LENGTH IN MILLIMICRONS
1. MMb
2. MMb + 1 ml H_2S water
3. MMb + 5 ml H_2S water
4. MMb + 10 ml H_2S water

Figure 2. Spectrophotometric curves obtained from dialyzed metmyoglobin treated with saturated H_2S water (Keilin's pigment).

Curves characteristic of metmyoglobin or cyanmetmyoglobin were not formed by the addition of the potassium ferricyanide and potassium cyanide to the Hoppe-Seyler pigment solution. Thus while the green pigment undergoes oxidation similar to that of reduced myoglobin to metmyoglobin, it is a stable myoglobin sulfur complex which cannot be converted to cyanmetmyoglobin. From this experiment it would appear that the Hoppe-Seyler pigment is not ordinarily found or developed in mixed pigment solutions since its formation eliminated the maxima (Figure 1) of the typical mixed pigment adsorption curve. Also, it fails to behave in a manner similar to the mixed pigments when treated with potassium ferricyanide and potassium cyanide.

In producing Keilin's sulfmetmyoglobin pigment, a dialyzed metmyoglobin solution containing only a very slight excess of potassium ferricyanide was treated with saturated hydrogen sulfide water. This resulted in an immediate reddening of the brown metmyoglobin solution. The red sulfmetmyoglobin pigment exhibited characteristic peaks at 545 and 580 mmc with a much stronger absorption band at 545 than at 580 mmc (Figure 2). This was in agreement with the findings of Keilin *(7)*. Addition of increasing amounts of from 1 to 5 ml of saturated hydrogen sulfide water resulted in increased formation of the sulfmetmyoglobin pigment. When 5 or 10 ml, an apparent excess of hydrogen sulfide water, were added, a slight maximum occurred at

625 mmc (Figure 2). Since the conversion of metmyoglobin to Keilin's sulfmetmyoglobin is a reversible reaction, a partial explanation might be that the excess hydrogen sulfide changes some of the metmyoglobin to reduced myoglobin. In the presence of limited oxygen, the reduced myoglobin may react with the oxidized sulfur to form a compound related to the green Hoppe-Seyler pigment, which has a maximum in the region of 618 mmc.

When potassium cyanide was added to the metmyoglobin solution treated with hydrogen sulfide water, the characteristic cyanmetmyoglobin curve was not obtained. The peak noted at 625 mmc was greatly increased, and the major peak at 545 mmc was decreased with increasing concentration of hydrogen sulfide (Figure 3). Addition of potassium cyanide might produce a favorable condition for irreversible formation of the Hoppe-Seyler pigment or some related compound. The presence of this type pigment could account for the increase in absorption at 625 mmc and would result in lowering of the major absorption peak of sulfmetmyoglobin at 545 mmc. The decline of the peak at 545 mmc may also be accounted for in part by the fact that the cyanide group partially replaces the sulfide group with the formation of cyanmetmyoglobin which has a maximum absorption band at 540 mmc. The cyanmetmyoglobin, however, absorbs less strongly ($\epsilon = 11.3$) in this region

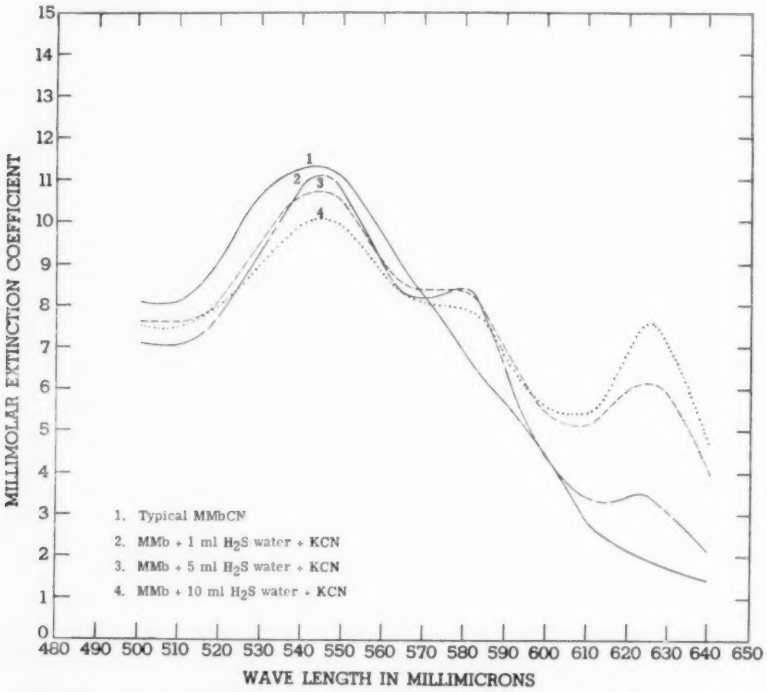
MILLIMOLAR EXTINCTION COEFFICIENT
WAVE LENGTH IN MILLIMICRONS
1. Typical MMbCN
2. MMb + 1 ml H2S water + KCN
3. MMb + 5 ml H2S water + KCN
4. MMb + 10 ml H2S water + KCN

Figure 3. Spectrophotometric curves obtained upon addition of KCN to H_2S water treated dialyzed metmyoglobin solutions.

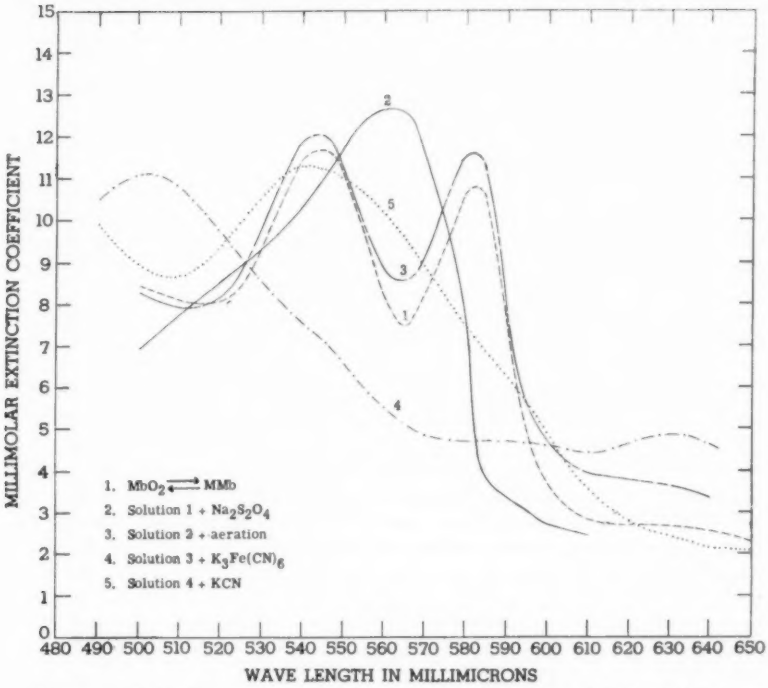
MILLIMOLAR EXTINCTION COEFFICIENT
WAVE LENGTH IN MILLIMICRONS
1. $MbO_2 \rightleftharpoons MMb$
2. Solution 1 + $Na_2S_2O_4$
3. Solution 2 + aeration
4. Solution 3 + $K_3Fe(CN)_6$
5. Solution 4 + KCN

Figure 4. Typical spectrophotometric curves obtained when mixed solutions were treated with $Na_2S_2O_4$.

than the sulfmetmyoglobin ($\epsilon = 12.3$), thus explaining the observed decrease in the reading at 545 mmc. While it is not impossible that the green pigment indicated by the peaks at 625 mmc (Figures 3, 4) is due to a porphyrin ring opening, such a reaction would not be expected under the conditions of this experiment.

The formation of an absorption peak at 625 mmc was not noted in any other phase of the present experiments. Neither would one be expected to occur even if small amounts of hydrogen sulfide were contained naturally in the meat extract. All pigment solutions except the dialyzed metmyoglobin solution used in the hydrogen sulfide water treatment and the solutions used in the cysteine treatments contained excess potassium ferricyanide when potassium cyanide was added. The excess potassium ferricyanide would prevent any hydrogen sulfide released by replacement with the cyanide radical from reducing small amounts of metmyoglobin to myoglobin. If reduced myoglobin were absent in the solution, the formation of a reduced Hoppe-Seyler type pigment would be prevented; and, therefore no peak would be noted in the 625 mmc region.

The presence of Keilin's sulfmetmyoglobin pigment might produce the type of systematic variation observed in estimates of metmyoglobin in mixed pigment solutions made at different wave lengths *(8)*. Since the method of

estimation of metmyoglobin is based on the assumption that a mixed pigment solution contains only oxymyoglobin and metmyoglobin in a state of equilibrium, the presence of a small amount of Keilin's pigment would have an influence on the estimated value of metmyoglobin. Although its absorption maxima at 545 and 582, and minimum at 562 mmc are in the same regions as those of oxymyoglobin, the extinction coefficients bear quite a different relationship to one another (Figure 2).

The effect of Keilin's pigment on the estimated concentration of metmyoglobin in mixed pigment solution can be studied by comparing its extinction coefficients with those of oxymyoglobin. While the extinction coefficients of sulfmetmyoglobin are not available, those for sulfmethemoglobin published by Drabkin *(3)* are adequate for use in this comparison. At 562 mmc the absorbance of Keilin's pigment ($\epsilon = 10.1$) is stronger than that of oxymyoglobin ($\epsilon = 8.5$). The increase in absorbance of light due to the presence of Keilin's pigment, would give an apparent low calculated value for the metmyoglobin present in the solution. At 582 mmc Keilin's pigment ($\epsilon = 10.4$) has an absorbance considerably less than that of oxymyoglobin ($\epsilon = 15.1$). The presence of Keilin's pigment would thus tend to decrease the absorbance of the solution resulting in a high estimate for the metmyoglobin content of the mixed pigment solution. At 545 mmc the sulfmetmyoglobin ($\epsilon = 12.3$) also absorbs less strongly than oxymyoglobin ($\epsilon = 14.6$), but the difference between the absorbance of the oxymyoglobin and Keilin's pigment is less pronounced at this wave length than that noted at 582 mmc. This would result in a high estimated value for metmyoglobin which would lie somewhere between the other 2 values.

Assuming the presence of only oxy- and metmyoglobin, estimates of the latter at different wave lengths, under conditions which should be favorable for complete metmyoglobin formation, varied from 79.0% at 564 to 92.4% at 582 mmc. This failure to obtain comparable estimates adds further support to the hypothesis of the presence of a third pigment form. Thus, if this pigment is responsible for the systematic variation, the highest estimate for metmyoglobin would be expected to be obtained at 582 mmc, with the value calculated from measurements at 562 mmc being the lowest. The previously noted systematic variation in metmyoglobin estimates *(8)* as well as those noted in the present experiments follow the above pattern.

The comparison of observed ratios at critical wave lengths, 544, 564 and 582 mmc with theoretical ratios for pure oxymyoglobin, metmyoglobin and sulfhemoglobin indicates that the ratio obtained for the typical mixed pigment curve at 544/564 mmc does not fall between the values for oxymyoglobin and metmyoglobin, but does fall between the values for oxymyoglobin and sulfmethemoglobin. Ratios obtained at 582/544 and 582/564 mmc would be possible for solutions of only oxymyoglobin and metmyoglobin, or for those containing a third pigment sulfmetmyoglobin. A study of the observed ratios for the mixed pigment solution shows that they are very similar to values calculated for several theoretical 3 pigment mixtures (Table 1).

Extinction coefficients for metmyoglobin obtained in this laboratory from mixed pigment solutions were consistently higher than published extinction coefficients for this compound *(2)*. Assumption of the presence of sulfmetmyoglobin in the presumably pure metmyoglobin solutions might explain part of the discrepancy in both extinction coefficients and ratios.

TABLE 1

Comparison of extinction coefficient ratios for theoretical pure and mixed pigment solutions with observed values

Solution composition			Ratios		
MbO_2	MMb	SMMb	544/564	582/564	582/544
%	%	%			
Theoretical Values					
100	0	0	1.72	1.78	1.03
0	100	0	1.60	0.91	0.57
0	0	100	1.22	1.03	0.84
70	10	20	1.58	1.56	0.98
60	25	15	1.61	1.53	0.95
55	30	15	1.60	1.50	0.94
50	25	25	1.54	1.45	0.93
50	35	15	1.59	1.47	0.92
Observed Values					
Average value for mixed pigment			1.59	1.51	0.95
Average value for MMb			1.43	0.90	0.62

As sulfmetmyoglobin is formed with the addition of increasing amounts of hydrogen sulfide water the ratios at 544/564, and 582/544 progress in the expected direction going from the typical metmyoglobin value and approaching the typical sulfmetmyoglobin value with the addition of 10 ml of hydrogen sulfide water. But the ratio 582/564 (Table 2) does not progress as expected. Study of the curves suggests that the addition of 5 ml of hydrogen sulfide is enough to approach the maximum formation of sulfmetmyoglobin (Figure 2). All 3 ratios support this conclusion.

With the addition of cysteine to metmyoglobin, the ratio picture is quite similar to that described above. With increasing additions of cysteine, all 3 ratios progress toward those of sulfmetmyoglobin (Table 2). It is possible that the spectrophotometric curve produced by treating metmyoglobin with cysteine may in part be the result of the fact that sulfhydryl groups reduce some of the metmyoglobin which then combines with oxygen to form oxymyoglobin. Both sulfmetmyoglobin and oxymyoglobin, if formed, would yield the observed typical cyanmetmyoglobin curve (Figure 6).

TABLE 2

Comparison of ratios for metmyoglobin and sulfmethemoglobin with those for metmyoglobin solutions treated with saturated hydrogen sulfide water or cysteine

Solution			Ratio		
MbO_2	MMb	SMHb	544/564	582/564	582/544
Theoretical Value					
0	100	0	1.60	0.91	0.57
0	0	100	1.22	1.03	0.85
Observed MMb solution			1.43	0.89	0.62
+ 1 ml H_2S water			1.32	1.02	0.77
+ 5 ml H_2S water			1.24	0.98	0.78
+10 ml H_2S water			1.23	0.98	0.78
+.004 mg cysteine			1.46	0.90	0.61
+.008 mg cysteine			1.44	0.96	0.66
+.020 mg cysteine			1.35	0.96	0.70
+.040 mg cysteine			1.39	1.03	0.74
+.080 mg cysteine			1.32	1.02	0.77

The effect of a third sulfur compound, sodium hydrosulfite, on the mixed pigment was studied. The addition of sodium hydrosulfite to the solution resulted in the rapid disappearance of the two maxima at 544 and 582 mmc. A maximum was formed at 562 mmc which is characteristic of reduced myoglobin (Figure 4). If the reduced myoglobin solution was aerated immediately the equilibrium between the oxymyoglobin and metmyoglobin was re-established quickly, yielding the typical mixed pigment curve. This curve differs from the original curve in that a slight elevation occurs, the elevation being more pronounced at 564 mmc than at 544 and 582 mmc. The increase in absorption at 564 mmc would not be due to reduced myoglobin since with aeration it is readily converted into the other two forms of the pigment. It appears that a small amount of some substance is present in the solution which can be reduced by sodium hydrosulfite giving a maximum at 562 mmc, and that this substance is not easily oxidized by shaking with oxygen. Cytochrome b would behave in this manner if present, but its presence is doubtful as Keilin *(7)* found cytochrome b to be rather difficult to extract from muscle tissue.

The addition of potassium ferricyanide and potassium cyanide to the sodium hydrosulfite-treated aerated solution formed the typical metmyoglobin and cyanmetmyoglobin curves respectively (Figure 4). Thus in an atmos-

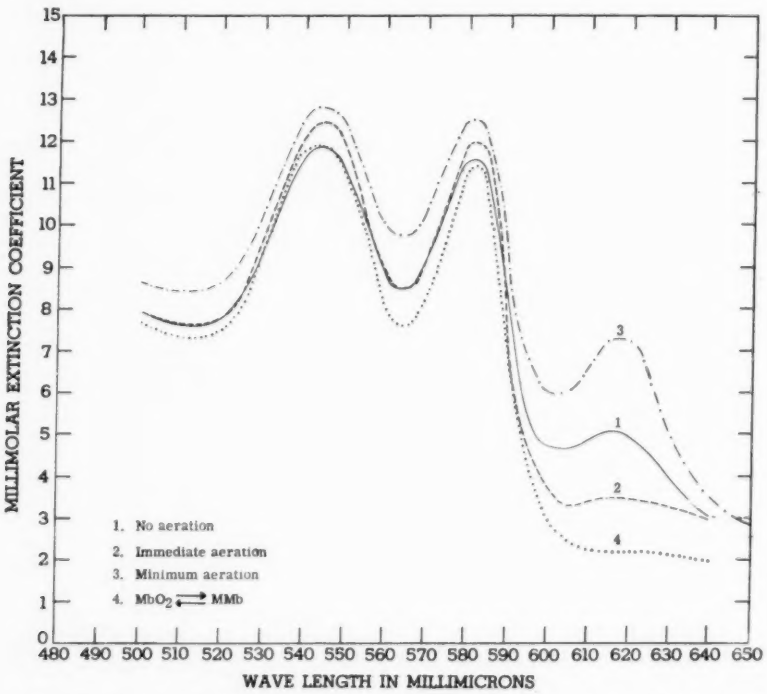

Figure 5. The effect of aeration on spectrophotometric curves from mixed pigment solutions treated with $Na_2S_2O_4$. All curves were read two hours after treatment.

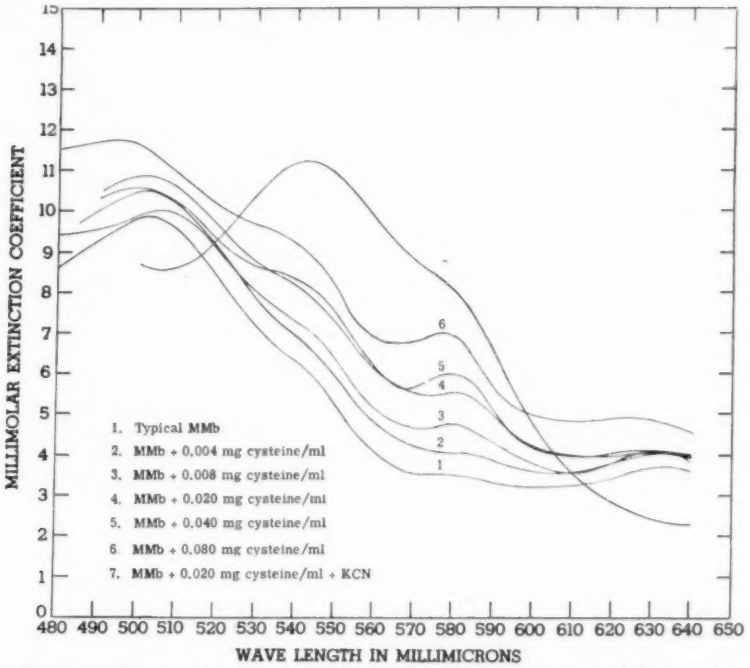
MILLIMOLAR EXTINCTION COEFFICIENT
WAVE LENGTH IN MILLIMICRONS
1. Typical MMb
2. MMb + 0.004 mg cysteine/ml
3. MMb + 0.008 mg cysteine/ml
4. MMb + 0.020 mg cysteine/ml
5. MMb + 0.040 mg cysteine/ml
6. MMb + 0.080 mg cysteine/ml
7. MMb + 0.020 mg cysteine/ml + KCN

Figure 6. Spectrophotometric curves obtained on addition of specified amounts of cysteine to metmyoglobin containing only a slight excess of $K_3Fe(CN)_6$.

phere containing a sufficient oxygen supply the only marked effect of sodium hydrosulfite on the mixed pigment is to convert it temporarily into reduced myoglobin which is again readily oxidized.

If no aeration of the sodium hydrosulfite treated sample occurred, the length of time required for the re-establishment of the oxymyoglobin, metmyoglobin equilibrium was greatly increased, but at the end of 2 hours the absorption curve was found to be quite similar to that of the sample aerated immediately after reduction. It was noted, though, that the absorbance for the non-aerated sample was slightly less at 544 and 582 mmc than for the immediately aerated sample. Also a slight peak in the non-aerated sample was noted to occur at 618 mmc (Figure 5). When oxygen was present in the solution in a limited supply, a greenish coloration was noted which resulted in a more pronounced absorption maximum at 618 mmc. A similar absorption maximum has been noted for solutions treated with H_2O_2 in the presence of a reducing agent *(4)*. This corresponds to the absorption maximum noted with the Hoppe-Seyler pigment. In order for the formation of Hoppe-Seyler pigment to occur, some oxygen is essential. The long standing of the reduced pigment in the presence of the reducing agent ($Na_2S_2O_4$) and oxygen appears in some manner to have provided favorable conditions for the formation of a sulfmyoglobin pigment. The slow oxidation of the reduced pigment was

found also to exaggerate further the elevation in the absorption minimum at 564 mmc.

SUMMARY

The possibility of the presence of a third pigment form in extracts of muscle tissue was investigated by studying the behavior of meat pigment extracts on addition of a number of sulfur-containing compounds: hydrogen sulfide gas, hydrogen sulfide water, sodium hydrosulfite, cysteine, glutathione and 1-amino-2-naphthol-4-sulfonic acid. No appreciable effects were obtained with the latter two compounds. It was possible to exaggerate the tendency toward systematic differences in both estimation of concentration of metmyoglobin and extinction coefficient ratios at critical wave lengths by the addition of hydrogen sulfide water or cysteine. Thus from these studies, it appears that the sulfur containing pigment described by Keilin may be present in mixed pigment solutions. This pigment seems to persist under conditions which presumably bring about complete conversion of pigments to metmyoglobin, but appears to be completely converted to cyanmetmyoglobin.

A number of inconsistencies in the spectrophotometric behavior of pigment solutions under the conditions of these experiments indicate further work should be done before evidence of the presence of a third pigment form can be considered conclusive.

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EFFECT OF ADDITION OF ANTIOXIDANTS TO FROZEN GROUND BEEF[a]

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(Manuscript received January 14, 1959)

In recent years antioxidants have been used to help protect foods from the off colors, flavors, and odors associated with oxidative rancidity *(1, 4, 6, 8)*. Several workers have found that antioxidants used in combination sometimes have a more pronounced protective effect than when used singly *(5, 7)*. Of the many antioxidants tested, ascorbic acid and its sodium salt have been found to be the most useful in preserving the color of fresh meat. Both compounds have also been found to aid the regeneration of the red color in frozen meat that has been thawed and exposed to air *(11)*. However, some workers have found these compounds may cause the meat to discolor, resulting in greening *(2, 3, 9)*.

The purpose of this study was to investigate the effect of several antioxidants, used singly and in combination with the antioxidant sodium ascorbate, on the color and flavor of ground beef preserved by freezing. The antioxidants studied were sodium ascorbate, nordihydroguaiaretic acid (NDGA), ethylenediamine tetraacetic acid (EDTA), sodium citrate, and dihydroxy quercetin.

EXPERIMENTAL METHODS

Good grade beef round with all visible connective tissue and fat removed was used for the study. The beef was ground three times through an electric grinder with a one-eighth inch plate. Six hundred gram portions of the ground beef were treated as shown in Table 1. The treatment solution was poured over the surface of the meat. The sample was mixed by hand for one minute. Fifty gram portions were weighed and wrapped in polyethylene terephthalate film with an overwrap of aluminum foil. The samples were stored at −23° C.

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TABLE 1

Constituents of solutions used in treatment of 600 grams of ground beef

Treatment	Physiological saline solution	Sodium Ascorbate	NDGA	EDTA	Sodium Citrate	Dihydroxy Quercetin
Control	12 ml					
Ascorbate	12 ml	1.8 g				
NDGA	12 ml		1.8 g			
Ascorbate + NDGA	12 ml	1.8 g	1.8 g			
EDTA	12 ml			1.8 g		
Ascorbate + EDTA	12 ml	1.8 g		1.8 g		
Sodium Citrate	12 ml				1.8 g	
Ascorbate + Citrate	12 ml	1.8 g			1.8 g	
Dihydroxy Quercetin	12 ml					1.8 g
Ascorbate + Quercetin	12 ml	1.8 g				1.8 g

The samples were evaluated while fresh and after 2, 4, and 6 weeks of storage. Four methods of evaluation were employed: subjective panel evaluations for color and flavor; spectrophotometric curves for the wave lengths 450 to 650 mmc; colored photographs; and thiobarbituric acid (TBA) determinations for rancidity.

The subjective panel was composed of 5 members of the Food Division, Home Economics Department. They evaluated the samples for color while frozen, after thawing, and after pan broiling. The cooked samples were evaluated for flavor. Ten point hedonic scales were used for scoring the samples as follows.

Score	Color evaluation	Flavor evaluation
1–2	extremely poor	undesirable
3–4	poor	slightly undesirable
5–6	fair	acceptable
7–8	good	moderately desirable
9–10	excellent	desirable

A Cary recording spectrophotometer with a reflectance attachment was used for the spectrophotometric determination on the 50 g ground meat samples. The meat was wrapped in polyethylene terephthalate film in order to obtain a smooth surface for the readings. Absorption curves were obtained for wave lengths from 450 to 650 mmc on both the frozen and the thawed samples.

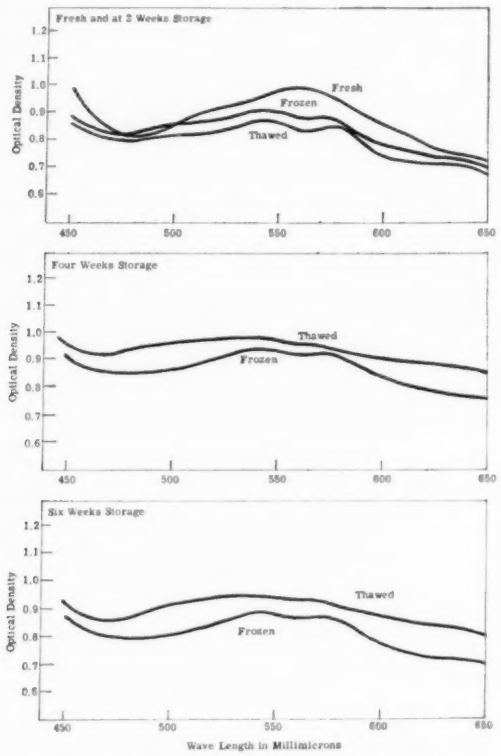

Figure 1. Absorbence curves for control sample.

Colored photographs were taken of the samples while frozen and after thawing. TBA determinations were made at each testing period to measure any rancidity that had developed. The method of Turner *et al.* was followed *(10)*.

RESULTS AND DISCUSSION

In general, the results of the spectrophotometric determinations, panel evaluations, and color photography substantiated each other (Table 2).

TABLE 2

Summary of color evaluation of thawed ground beef patties which had been treated with antioxidants and held in frozen storage up to six weeks

Treatment	Storage time in weeks	Panel rating	Type spectra curve	Color description
Control	0	excellent	reduced	dull purplish red
	2	fair	oxy + met	brownish red
	4	fair	reduced + met	brownish purple
	6	poor	met	brown
Ascorbate Cointrol	0	good	reduced	dull purplish red
	2	excellent	oxy	red
	4	good	reduced + oxy	bright purplish red
	6	excellent	reduced + oxy + met	brown purplish red
NDGA	0	fair	reduced	dull purplish red
	2	fair	oxy + met	brownish red
	4	poor	met	brown
	6	poor	met	brown
Ascorbate + NDGA	0	excellent	reduced	dull purplish red
	2	good	reduced + met	brownish purple
	4	good	reduced + oxy + met	brown purplish red
	6	good	oxy	bright red
Citrate	0	good	reduced	dull purplish red
	2	fair	met	brown
	4	fair	met	brown
	6	fair	met	brown
Ascorbate + Citrate	0	good	reduced + oxy	bright purplish red
	2	good	reduced + met	brownish purple
	4	good	reduced + oxy + met	brown purplish red
	6	good	reduced + oxy + met	brown purplish red
EDTA	0	poor	reduced + met	brownish purple
	2	fair	oxy + met	brownish red
	4	poor	met	dark brown
	6	poor	met	dark brown, some greening
Ascorbate + EDTA	0	good	reduced	dull purplish red
	2	excellent	oxy + met	brownish red
	4	good	reduced	dull purplish red
	6	good	reduced + met	brownish purple
Dihydroxy Quercetin	0	good	met + oxy	reddish brown
	2	fair	reduced + met	brownish purple
	4	poor	met	mottled reddish brown
	6	poor	met + oxy	reddish brown, some greening
Ascorbate + Dihydroxy Quercetin	0	good	oxy + reduced	bright reddish purple
	2	good	met	brown
	4	fair	reduced + oxy	bright purplish red
	6	good	reduced + oxy	bright purplish red

The color visible to the eye appears to be a result of concentration of the pigment components in the mixture. The spectrophotometric absorbances of the frozen samples were usually less than those of the corresponding thawed samples. This was probably caused by a slight condensation of moisture on the surface of the film during the determinations with the frozen samples. The color of frozen samples was judged as fair to good in every instance. The greatest variations in color were noted in the thawed samples. However, after cooking, no color differences due to treatment were detected.

Control and ascorbate control samples in the fresh state gave curves typical of reduced myoglobin and were a rather dull reddish purple (Figure 1, 2). The frozen samples changed very little throughout the period of storage, but the thawed control sample changed to a brown color and metmyoglobin type curve more rapidly than the ascorbate control.

Samples containing ascorbate showed no evidence in either the fresh or frozen state of the presence of metmyoglobin, a brown pigment. However, citrate, EDTA, NDGA, and dihydroxy quercetin used alone all tended toward the formation of metmyoglobin much more rapidly than

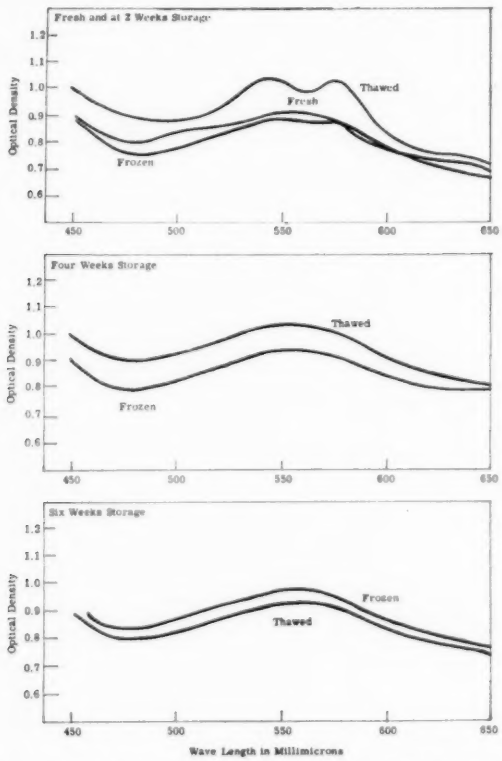

Figure 2. Absorbence curves for the sodium ascorbate sample.

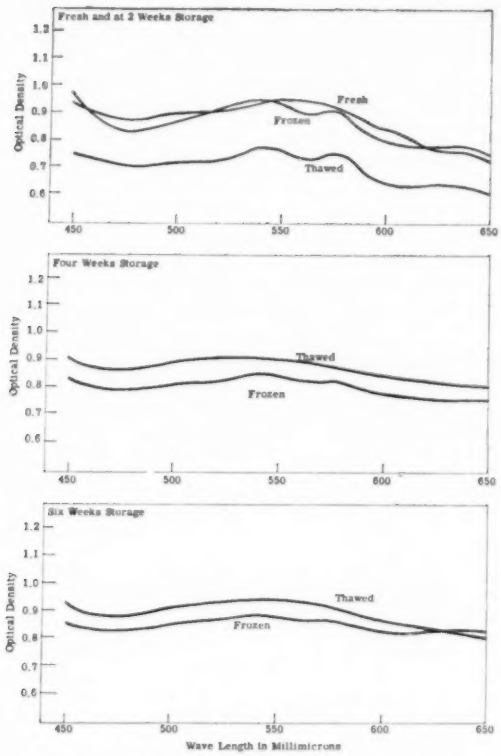

Figure 3. Absorbence curves for the NDGA sample.

when ascorbate was present. In the absence of ascorbate the color of the samples was rated only fair by the end of 2 weeks' storage, and poor in most cases by 4 weeks. The typical metmyoglobin curve shown by these compounds is illustrated by the curve for NDGA in Figure 3.

Results of the subjective panel ratings for flavor showed that the samples containing NDGA alone or in combination with ascorbate scored consistently low, and were rated undesirable. All other samples were rated acceptable or higher but decreased in acceptability with storage time.

Although the TBA tests gave values that varied appreciably (from 0.0055 for dihydroxy quercetin at 4 weeks of storage to 0.3370 for NDGA at 4 weeks) none of the values were high enough to indicate rancidity. This was to be expected since the storage period had been relatively short, and since all visible fat had been removed giving a ground meat with a low fat content.

SUMMARY

In general, ascorbate used alone or in combination with each of the other antioxidants maintained a good color in the thawed samples through-

out the six weeks of frozen storage. The greening reported by early workers was not observed under the conditions of these experiments. The antioxidants other than ascorbate failed to protect the color of meat during frozen storage under the conditions of these experiments. Thus, sodium ascorbate alone appears to be the most satisfactory additive for color preservation in frozen meat. With the exception of NDGA, which contributed an undesirable flavor, the antioxidants appeared to have little effect on the flavor of the meat.

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STUDIES ON ASCORBIC ACID RETENTION IN FROZEN JUICE, SEGMENTS, AND WHOLE ORANGES

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During the course of some investigations on cloud retention in single-strength orange juice, it was observed that the samples lost ascorbic acid during extended storage at 0° F. The investigations were then expanded to include observations on the retention of ascorbic acid in frozen segments and whole oranges, in addition to single-strength juice.

Orange juice which is held at sub-freezing temperatures at the present time is usually stored as the concentrate and it has been reported that little ascorbic acid is lost under these conditions *(1, 2, 3, 4, 5)*. Marshall, Hays, Fellers and DuBois *(3)* believed that loss of ascorbic acid could be attributed to oxidation which was enhanced by the presence of air in head space of the container. Evenden and Marsh *(2)*, working with single-strength orange juice, found that the loss of vitamin C was reduced by deaerating the juice before canning and freezing. In the work now being reported it was not practicable to deaerate the juice but it was felt that oxidation might be reduced by storing the oranges as segments or as whole fruits. Information thus obtained should be of value both for storing and for transportation of frozen citrus products.

EXPERIMENTAL

Two crates of Valencia oranges were obtained from the Chase Groves in Windemere, Florida, and a third was purchased on the wholesale market in Pittsburgh. A whole crate of oranges was employed in each of 3 successive experiments. Upon arrival at the laboratory the oranges were peeled, removing both flavedo and albedo carefully so as not to rupture the juice vesicles. The peeled oranges were divided into 3 lots. In lot A, the fruits were separated into segments which in turn were macerated in a Waring blender. The juice was filtered through cheese cloth and transferred to one-pint waxed freezer cups. Lot B consisted of orange segments which were stored in the same type of container. Lot C consisted of whole, peeled oranges. These were stored in ½ gal cardboard containers designed for deep-freeze storage. All lots were placed in storage at 0° F (−17.7° C).

Once a month a sample from each lot of frozen material was removed from storage. The samples were thawed out by holding at 5° C for 24 hr. After thawing, the segments and whole oranges were juiced as described above for lot A. Ascorbic acid determinations were made on the juice from each lot by combining equal volumes of 3% metaphosphoric acid and juice, then titrating with 2,6-dichlorobenzenoneindophenol.

RESULTS AND CONCLUSIONS

Tables 1, 2 and 3 show the monthly changes in ascorbic acid content of samples stored as juice, segments and whole fruits. It will be observed that a gradual loss of ascorbic acid occurred in all lots over a six-month period. The rate of loss was somewhat greater in the third experiment than in the first two. The total loss at the end of 6 months was rather uniform irrespective of the manner in which the product had been stored. The third experiment appears as an exception in this regard but there was so much variation in

TABLE 1

Ascorbic acid content of juice, segments, and whole Valencia oranges stored at ° F (storage period started February 17, 1956)

Months stored at 0° F	Ascorbic acid (mg per 100 ml of juice)		
	Juice	Segments	Whole oranges
0	59.2	59.2	59.2
1	54.3	50.0	51.6
2	38.8	52.4	47.6
3	53.6	49.1	52.7
4	45.9	45.1	47.7
5	45.7	44.9	47.4
6	42.5	45.4	42.5

TABLE 2

Ascorbic acid content of juice, segments, and whole Valencia oranges stored at 0° F (storage period started June 6, 1956)

Months stored at 0° F	Ascorbic acid (mg per 100 ml of juice)		
	Juice	Segments	Whole oranges
0	44.4	44.4	44.4
1	39.6	42.6	41.7
2	.....	.....	.....
3	40.0	39.1	39.1
4	38.0	37.3	39.8
5	36.7	33.3	35.5
6	34.5	31.7	34.9

samples that differences between juice and segments or whole fruits are not significant. When results of all 3 experiments are averaged the retention of ascorbic acid over the 6-month period for the 3 lots was as follows: juice, 68.1% ; segments, 69.4% ; and whole oranges, 73.7%.

The reason for these losses in storage is not clear. There are several possibilities. Doubtless some ascorbic acid was lost during maceration of the segments in the blender. This would not account, however, for the month-by-month loss by the juice but should be reflected in the first sample only. A certain quantity of ascorbic acid may have been lost during thawing although

TABLE 3

Ascorbic acid content of juice, segments, and whole Valencia oranges stored at ° F (storage period started April 12, 1957)

Months stored at 0° F	Ascorbic acid (mg per 100 ml of juice)		
	Juice	Segments	Whole oranges
0	58.4	58.4	58.4
1	51.5	55.1	57.1
2	47.4	49.4	50.5
3	45.8	47.5	39.1
4	34.3	37.1	39.9
5	35.0	38.1	48.4
6	31.9	35.1	41.2

the temperature never rose above 5° C. If all losses occurred while the product was kept in the frozen state it does not seem likely that it was the result of enzyme action. Whatever the case, there appeared to be no advantage in storing oranges as segments on whole peeled fruits, rather than as juice.

SUMMARY

Valencia oranges from Florida were prepared as whole peeled fruits, segments, and juice and stored in freezer cups at 0° F for six months. Analyses at the end of each month of storage showed a steady loss in ascorbic acid for whole fruits, segments, and juice. No significant differences were found in rate of loss whether the material was stored as juice, segments or whole peeled oranges.

Acknowledgments

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METHODS OF ANALYSIS OF SOLUBLE CARBOHYDRATES AND PECTIC SUBSTANCES OF CITRUS FRUITS[a]

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(Manuscript received February 13, 1959)

During an extended investigation into the seasonal changes in the chemical constituents of citrus fruit tissues, it was found that the carbohydrates had to be separated into soluble and insoluble fractions before chemical methods could be subsequently used for determining the various constituents in the fruits. It is well known that 80% alcohol is used to separate the colloidal and noncolloidal carbohydrate and nitrogen compounds in the analysis of plant materials. The alcohol-soluble fraction of plant tissues consists mainly of mono- and disaccharides, which, with the starch and dextrins, serve as readily available sources of energy for the living cells. The alcohol-insoluble solids include the cell-wall or structural components, such as cellulose, lignin, proteins, pectic substances, and hemicelluloses, which are closely related in the plant, especially the last two.

The composition and nature of citrus fruits are such that they afford many interesting problems of research for the plant biochemist. The peel, which is composed of two distinct portions, the flavedo or epicarp and the albedo or mesocarp, is easily separated from the pulp, which is the edible portion of the fruit. The flavedo, the outer portion of the peel, is composed chiefly of carotenoid pigments, vitamins, and essential oils. The albedo, the spongy inner portion of the peel, is composed chiefly of celluloses, soluble carbohydrates, pectic substances (protopectin and pectin), flavonoids, amino acids, and vitamins.

On the other hand, the pulp of citrus fruits, unlike that of most edible fruits, is divided into segments (carpels), the walls of which are not readily permeable; and each segment is composed of hundreds of units (vesicles, juice sacs), the walls of which are still more impermeable, especially laterally. The vesicles or juice sacs which are located within the segments are composed of celluloses, hemicelluloses, protopectin, pectin, sugars, flavonoids, amino acids, vitamin C, mineral salts, and other nutrients. The juice of the edible portion of citrus fruits is located in the vesicles. The soluble constituents in the juice are composed of soluble carbohydrates (glucose, fructose, and sucrose), organic acids (chiefly citric acid), vitamin C, vitamin B complex, mineral salts, a small concentration of pectic materials, and many other nutrients.

The results presented in this paper are concerned with the schematic arrangements used in the separation and fractionation of the peel, pulp, and juice of citrus fruits. These schemes have been applied very successfully in the separation of the pectic substances from the soluble carbohydrates and other soluble materials so that a biochemical study can be made of the different forms of pectic materials occurring naturally in citrus fruits.

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[a] Paper No. 1176, University of California Citrus Experiment Station, Riverside, Calif.

RESULTS AND DISCUSSION

Alcohol-soluble fraction of citrus peel. The data in Figure 1 show diagramatically the scheme used to extract (with 80% ethyl alcohol) the alcohol-soluble fraction of ground citrus peel. This same procedure is also applicable to both the pulp (vesicles) and the juice of citrus fruits. The chief components of the alcoholic extract of citrus peel are the soluble carbohydrates and organic acids. Aside from the prolamin type of protein, the chief nitrogen constituents in the extract are the amino acids and dipeptides, which are the compounds that yield amid and amino nitrogen. Inorganic nitrogen compounds also occur in the extract.

The alcoholic extract also contains the essential oils, pigments, flavones, and bioflavonoids of the peel. Most of the essential oils are evaporated on a

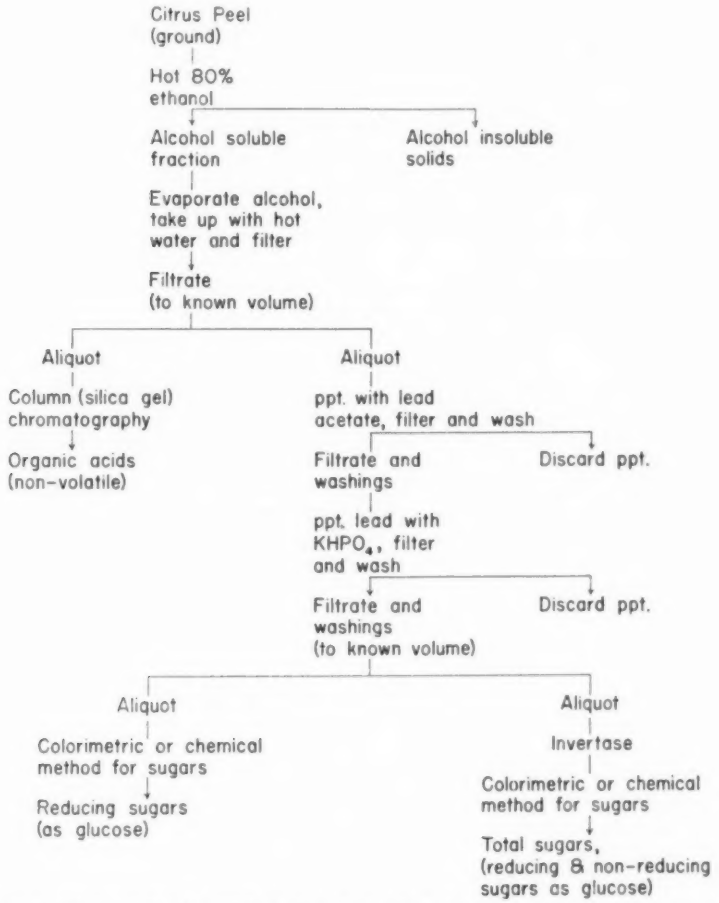

Figure 1. Diagram showing method of analysis of alcohol-soluble fraction of citrus peel.

steam bath with alcohol during the reduction of the extract to a small volume. The resulting aqueous phase is dissolved to known volume in water ready for analysis.

In Figure 1 the emphasis in the diagram is on the analysis of aliquot portions of the filtrate for nonvolatile organic acids and soluble carbohydrates. The literature contains many quantitative chemical procedures involving methods of analyzing for organic acids. In the use of column chromatography the acids are absorbed upon columns such as silica gel or Dow ion-exchange resin and then eluted with a solvent that permits their collection as separate fractions for further analysis. The use of the very sensitive paper chromatography involves the separation of the various organic acids from a spot of application on paper by developing with an appropriate solvent and then detecting them with an indicator.

The mono- and disaccharides are determined on aliquot portions of the cleared and deleaded filtrate (Figure 1). The monosaccharides or reducing sugars can be determined colorimetrically or by one of the volumetric reduction methods. Since other reducing substances are in the filtrate which would give erroneous carbohydrate values, a method that is reliable and specific for glucose and fructose, such as paper chromatography, should be used.

A separate aliquot portion of the deleaded filtrate is used for determining the total soluble sugars. The disaccharide sucrose is hydrolyzed with the enzyme invertase to glucose and fructose. The total reducing sugars determined on the inverted solution and expressed in terms of glucose represent the mono- and disaccharides of the sample. Again these carbohydrates are more specifically determined by means of paper chromatography.

The material soluble in 80% ethyl alcohol averaged 57.31% of the dry weight of Valencia orange whole peel and 55.19% of that of Navel orange albedo (Table 1). These values are higher than those reported for lemon peel but are lower than those for grapefruit peel. Total sugars (as glucose) accounted for 55.40% of the alcohol-soluble solids in Valencia peel and for 72.69% of that in navel albedo (Table 2). On the dry-weight basis, these values became 31.71% and 40.11%, respectively. The amount of reducing sugars was considerably greater than that of sucrose; the ratio was approximately 3.5:1 in Valencia peel and 7.0:1 in navel albedo. The alcohol-insoluble fraction contained substances which liberated a small amount of carbon dioxide on hydrolysis with 19% hydrochloric acid. Mean values were 0.52% for Valencia peel and 0.63% for navel albedo. This relatively small amount of carbon dioxide was probably supplied by a very low polymer pectic material and free galacturonic acid dissolved by the hot 80% alcohol during the extraction process.

Alcohol-insoluble solids of citrus peel. Although the alcohol-insoluble solids contain the proteins, pectic substances, and other high molecular weight compounds, the diagram of Figure 2 is concerned only with the separation and analysis of the pectic substances. As the alcohol-insoluble solids of the peel of mature citrus fruits contain practically no starch, it is highly suitable as a source of pectic material for scientific investigations.

The solids which are insoluble in 80% alcohol, and which, as already noted, are composed chiefly of the structural components of the cells, amounted to 42.69% and 44.81% of the dry weight of Valencia whole peel

TABLE 1

The carbon dioxide and methoxyl contents in relation to the anhydrogalacturonic acid of alcohol-insoluble solids (A.I.S.) of citrus fruit tissues

Fruit tissue	Dry weight[1]	Alcohol-soluble solids[2]	Alcohol-insoluble solids[2]	Total CO_2[3]	Methoxyl	Anhydro-galacturonic acid
	%	%	%	*(% A.I.S.)*	*(% A.I.S.)*	*(% A.I.S.)*
Valencia orange:						
Peel		57.31	42.69	8.19	4.81	32.76
Pulp vesicles	9.51	90.13	9.87	6.02	2.83	24.08
Navel orange:						
Peel albedo		55.19	44.81	7.70	4.47	30.80
Pulp vesicles	12.25	84.44	15.56	6.72	3.95	26.88
	9.22	84.85	15.15	5.50	3.10	22.00
Grapefruit:						
Peel	18.57	66.31	33.69	10.44	5.72	41.76
Pulp vesicles	10.70	92.61	7.39	7.08	3.40	28.32
Lemon:						
Peel albedo	22.34	40.00	60.00	7.91	5.09	31.64
	19.10	37.26	62.74	8.59	5.88	34.36
Pulp vesicles	10.06	86.37	13.63	8.47	4.71	33.88

[1] Values calculated on fresh-weight basis.
[2] Values calculated on dry-weight basis.
[3] A.I.S. is abbreviation for alcohol-insoluble solids.

TABLE 2

Analysis of alcohol-insoluble solids (A.I.S.) and of alcohol-soluble solids (A.S.S.) of orange peel and albedo

Sample no	Alcohol-insoluble solids	Alcohol-soluble solids	CO_2 produced on hydrolysis with 19% HCl				Methoxyl in A.I.S.[1]	Ratio, methoxyl to CO_2 in A.I.S.	Methylation	Water-soluble sugars (as glucose) in alcoholic extract (% dry weight)			Total sugars
			Alcohol-insoluble solids		Alcohol-soluble solids								
			Dry weight[1]	A.I.S.[1]	Dry weight	A.S.S.				Reducing	Sucrose	Total	
	(% dry weight)	(% dry weight)	%	%	%	%	%		%				(% A.S.S.)
Valencia orange whole peel													
1	42.63	57.37	3.56	8.34	0.284	0.495	4.85	0.581	82.53	25.32	6.93	32.25	56.21
2	44.41	55.59	3.60	8.11	.282	.507	4.80	.592	84.09	24.70	7.80	32.50	58.46
3	41.02	58.98	3.33	8.11	0.322	0.546	4.77	0.588	83.52	23.78	6.61	30.39	51.53
Mean	42.69	57.31	3.50	8.19	0.296	0.516	4.81	0.587	83.38	24.60	7.11	31.71	55.40
Navel orange albedo													
1	44.84	55.16	3.51	7.83	0.360	0.653	4.40	0.562	79.83	36.17	4.24	40.41	73.26
2	44.70	55.30	3.46	7.74	.368	.665	4.54	.586	83.24	34.82	4.83	39.65	71.70
3	44.90	55.10	3.38	7.53	0.318	0.577	4.46	0.592	84.09	34.80	5.48	40.28	73.10
Mean	44.81	55.19	3.45	7.70	0.349	0.632	4.47	0.580	82.39	35.26	4.85	40.11	72.69

[1] Calculated on ash-free, moisture-free basis.

and of navel albedo, respectively (Table 1). The alcohol-insoluble solids of grapefruit and lemon peels amounted to 33.69 and 60.00% of their respective dry weights. In all instances the peel has a greater amount of alcohol-insoluble solids than either the pulp or the vesicles. This material is well suited for a study of the polyuronide fraction, as it is free of most soluble carbohydrates and pigments and of a considerable portion of the ash. The uronic acids yield 22.50% of carbon dioxide on hydrolysis. The pectic substances all contain galacturonic acid as a basic unit; therefore, the amount of carbon dioxide produced by hydrolysis with 19% hydrochloric acid should be a fair measure of the total pectic substances.

The total pectic material in the alcohol-insoluble solids may be determined from the carbon dioxide produced in decarboxylation of the galacturonic acid. The decarboxylation process is performed by boiling a known aliquot of the sample with either 12 or 19% hydrochloric acid *(2, 3)*. During the hydrolysis, the galacturonic acid decomposes into furfural, carbon dioxide, and water.

$$C_6H_{10}O_7 \longrightarrow C_5H_4O_2 + CO_2 + 3H_2O$$

In this reaction the yield of carbon dioxide is quantitative and it is a characteristic of the galacturonic acids. The percentage of carbon dioxide in the alcohol-insoluble solids, multiplied by four, is equivalent to the total galacturonic acid anhydride of the sample (Table 1). This means that the total pectic substances of the sample cannot be greater than that equivalent to the carbon dioxide experimentally produced, based, of course, on the assumption that all the carbon dioxide is derived from the pectic materials. If all the sugars have not been removed in the alcohol extraction, they will contribute nonuronide carbon dioxide upon hydrolysis with 19% HCl.

For determination of the methoxyl groups a separate aliquot sample is used for the saponification reaction with NaOH, according to the method of Romeo *(4)*. Knowing the galacturonic acid equivalent of the carbon dioxide and methoxyl contents of the sample, the degree of methylation of the pectic substances in the alcohol-insoluble solids can be estimated to a fair degree of accuracy (Table 2).

All pectic substances contain methoxyl (OCH_3) in varying amounts, depending on the source, method of preparation, etc. The methoxyl content of a completely esterified polygalacturonide would be 16.32%. Pectic substances containing such a high concentration of methoxyl have not as yet been isolated, but the figure is useful in calculating the degree of esterification of any given pectic sample. Pectic material from various sources yields, on the average, about 11% methoxyl on a moisture-free, ash-free basis. Since methoxyl is easily determined by saponification with sodium hydroxide, a rapid method is available for the approximate determination of pectic material. The uronic acid carboxyl groups of pectic substances are free for salt formation or esterification, but the hemicelluloses do not exhibit acidic properties when treated with alkali at room temperatures. The alcohol-insoluble solids of Valencia whole peel and of navel albedo yielded 4.81% and 4.47% (mean values) methoxyl, respectively (Table 1).

The ratio of methoxyl to carbon dioxide in a fully methylated galacturonide would be 31 : 44, or 0.704. Ratios for the alcohol-insoluble solids of Valencia whole peel and of navel albedo, as calculated from the data in

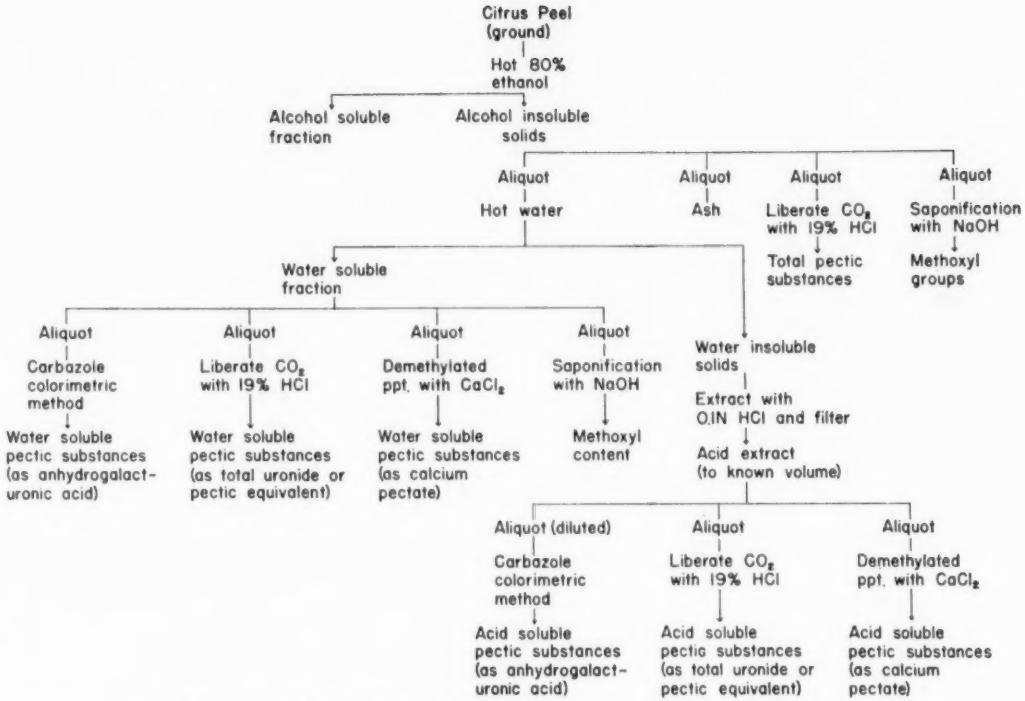

Figure 2. Diagram showing method of analysis of alcohol-insoluble solids of citrus peel.

Table 2, are 0.587 and 0.580, respectively. The relatively low methoxyl content and the absence of free acidity indicate that some of the carboxyl groups are combined with the cations of the alcohol-insoluble fraction. The degree of methylation of the total carboxyl groups can be calculated by the equation $0.587 \times 100/0.704 = 83.38\%$ for Valencia whole peel. The corresponding value for navel albedo is 82.39%.

Another way of obtaining the degree of methylation is by comparing the determined methoxyl with that equivalent to the total carbon dioxide. For the Valencia samples the mean carbon dioxide was 8.19%; this amount multiplied by 0.704 gives 5.76% equivalent methoxyl. The amount determined from the data in Table 1 is 4.81%. The simple calculation $4.81 \times 100/5.76 = 83.50\%$ gives the degree of methylation. The corresponding value for navel albedo is 82.47%. These percentages are in good mathematical agreement with the values given in the preceding paragraph.

Known samples of the powdered alcohol-insoluble solids are extracted with hot water, either on a filter paper or by mixing with water and centrifuging. The water-soluble fraction is diluted to known volume, and aliquot portions are taken for the determination of the pectic substances by various methods. An aliquot of the water-soluble fraction is diluted to sufficient volume to fall within the range of the carbazole method. An aliquot of this is then heated in a boiling-water bath with concentrated sulfuric acid to develop the necessary intermediates. The carbazole solution is added to this mixture and the color developed at room temperature for a definite period of time. The amount of color developed is then read in a spectrophotometer and compared with a calibration curve derived from known concentrations of galacturonic acid. The value obtained is in terms of galacturonic acid anhydride.

A second aliquot of the water extract was treated with 19% HCl to liberate the total uronide carbon dioxide from the extracted pectic materials. By using the proper conversion factor, the carbon dioxide values can be converted to the water-soluble pectic substances expressed as galacturonic acid, total uronide, or pectic equivalent.

A third aliquot of the water extract can be used to determine the water-soluble pectic substances, as calcium pectate, by the Carré and Haynes procedure *(1)*. This procedure involves essentially the demethylation of the pectic materials in solution made alkaline with NaOH. After standing for complete hydrolysis, the solution is subsequently made acid with acetic acid and, finally, the pectic substances are precipitated as calcium pectate with M $CaCl_2$ solution. By using a separate aliquot of the water extract, the total methoxyl content can be quantitatively determined by saponification with NaOH, and can be subsequently correlated with the total galacturonide content of the water extract as calculated from the carbon dioxide produced on hydrolysis with 19% HCl.

The residue (water-insoluble solids) that remains after the extraction of the water-soluble pectic substances is used to determine the protopectin and pectate or acid-soluble pectic substances from the alcohol-insoluble solids by hydrolysis, several times, with a weak concentration of HCl. The hydrolysates are diluted with water to standard volume, and aliquot samples are taken for the determination of pectic substances by the methods already

described for the water-soluble pectic materials. The total pectic substances of the samples are therefore composed of the sum of the water-soluble and acid-soluble pectic materials. The experimental values on the water- and acid-soluble pectic substances of citrus fruit tissues are published elsewhere *(5, 6, 7, 8)*.

SUMMARY

Methods are described for the separation and analysis of the carbohydrate fractions of the peel, pulp, and juice of citrus fruits. Citrus peel was the test material used in these experiments, but the principles and methods are equally applicable to the pulp, vesicles, and juice.

After extraction of the tissues with 80% ethyl alcohol, the carbohydrates were separated into alcohol-soluble and alcohol-insoluble fractions. A diagram is given for the separation and analysis of the nonvolatile organic acids and the soluble mono- and disaccharides (glucose, fructose, and sucrose) from the other constituents of the alcohol-soluble fraction. The amino acids are in this fraction and they also can be quantitatively determined by column and paper chromatography.

A diagram is given for the separation and analysis of the alcohol-insoluble solids, with special reference to the fractionation of the pectic substances of citrus peel. The alcohol-insoluble solids contain the proteins, pectic substances, and other high molecular-weight compounds. The water-soluble materials were extracted from the alcohol-insoluble solids, and from this extract the water-soluble pectic substances were subsequently determined on aliquot samples by the carbazole colorimetric method, total uronide CO_2 and the calcium pectate methods. The protopectin and pectate or acid-soluble pectic substances are extracted with dilute hydrochloric acid solution from the water-insoluble residue of the alcohol-insoluble solids, and the analyses for pectic substances are made by the methods described for the water-soluble fraction.

Experimental data presented show carbon dioxide and methoxyl contents in relation to the anhydrogalacturonic acid of the alcohol-insoluble solids of citrus fruit tissues, and certain analytical data are presented on the alcohol-insoluble and alcohol-soluble fractions of Valencia and Navel orange peels.

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